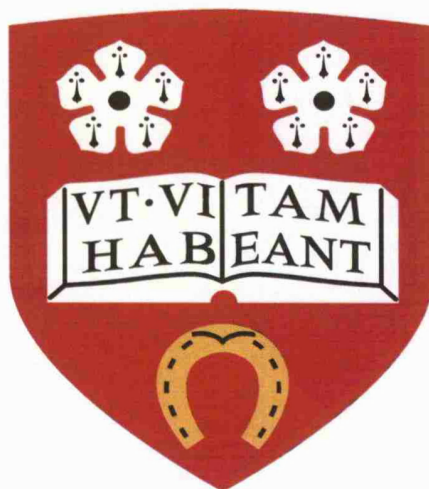


**PRE-CLINICAL DEVELOPMENT OF OXIDATIVE
DNA ADDUCTS AS BIOMARKERS FOR THE
CHEMOPREVENTION OF PROSTATE CANCER**



**THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF MEDICINE
AT THE UNIVERSITY OF LEICESTER**

BY

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JULY 2009

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ABSTRACT

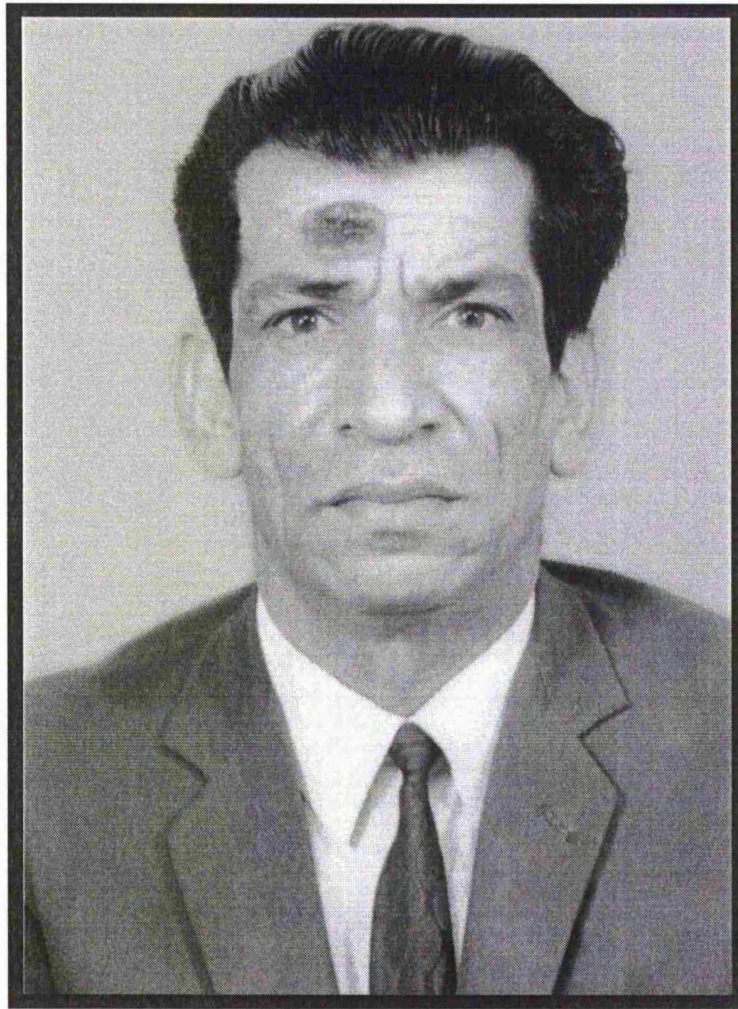
Reactive oxygen species (ROS) are mechanistically implicated in the development and progression of prostate cancer. Intracellular ROS may cause oxidative DNA damage, resulting in the formation of adducts, such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) and the cyclic pyrimidopurine N-1, N-2 malondialdehyde-2'-deoxyguanosine (M₁dG). These adducts have been associated with carcinogenesis, genomic instability and clonal evolution. It has previously been shown *in vitro* that testosterone and dihydrotestosterone (DHT) increase ROS levels in the androgen-sensitive, human prostate cancer cell line, LNCaP.

We tested two hypotheses using human prostate cancer cells grown *in vitro* and in a xenograft nude mouse model. 1) Levels of oxidative DNA adducts increase in LNCaP cells treated with DHT. 2) Flutamide, a competitive androgen receptor antagonist, prevents DHT-induced changes. The levels of M₁dG and 8-oxo-dG adducts were determined by immunoslot blot and liquid chromatography-tandem mass spectrometry, respectively. M₁dG and 8-oxo-dG adduct levels were significantly higher than control levels in LNCaP cells exposed to physiological levels of DHT. This effect was also concentration-dependent. Pre-treatment with flutamide completely prevented increases in oxidative DNA adducts.

In the athymic nude mouse LNCaP xenograft, tumour levels of M₁dG were decreased by 46% in flutamide-treated animals compared with controls. Flutamide also inhibited the growth of LNCaP tumours and this correlated with a reduction in serum Prostate Specific Antigen (PSA) levels.

The changes in oxidative DNA adduct levels *in vitro* and *in vivo* suggest that oxidative DNA adducts may serve as potential biomarkers of the efficacy of androgen manipulation in chemoprevention trials. Furthermore, a secondary finding of the study suggests that flutamide may be considered as a chemopreventive agent for prostate cancer. These adducts may serve as biomarkers of such a trial.

To My Late Dad



Prag Raj Pathak (1928-1995)

“If I have seen further than others, it is because I have stood on the shoulders of giants”

Sir Isaac Newton, 1675

ACKNOWLEDGEMENTS

Firstly, I would like to thank the Prostate Research Campaign (UK) for funding my first year salary.

I would like to thank my supervisors, Professor Andy Gescher, Dr Ricky Sharma, Professor Kilian Mellon and Professor Will Steward. They have been fully supportive both during my research and writing up of my thesis.

I would like to express my gratitude to Dr Richard Verschoyle for his help and support of my *in vivo* experiments. I would like to thank Dr Helen McCarthy (Queen's University, Belfast, Northern Ireland) and Dr Hong Cai for their help and advice in creating the xenograft model. I acknowledge the assistance of Dr Paul Garrick and Dr Peter Greaves in the determination of serum PSA levels and histological assessment, respectively. Thanks also go to the staff of the animal house for maintaining the mice.

Special thanks go to Dr Raj Singh for his help and contribution with the HPLC / Mass Spectrometry in the analysis of the oxidative DNA adducts.

Regarding my *in vitro* experiments, I would like to thank Carol and Janet for all their support. Carol allowed me exceptional access to her meticulously clean cell culture facilities when ours were temporarily unavailable. I was notorious for leaving my laboratory ordering until the last minute, however, despite being inconvenienced, Janet would kindly accept my order forms.

I would like to express my sincere thanks to all members of the Cancer Biomarkers and Molecular Medicine group for their support, particularly, Stephanie, Stewart, Giuseppe, Hari, Darren, Ketan, Dave and Tim.

I would like to thank Professor Nick London and Professor Sir Peter Bell for their support and encouragement, both as a medical student and as a surgical trainee. They laid the foundations of my surgical career.

Finally, I would like to dedicate my thesis to my late father, Prag Raj. I cherish the love and special moments we shared. I thank him for his lifelong sacrifices made to better our lives. Thanks also go to my sister Nav Kiran, my wise and considerate brother-in-law Rajinder, my niece Sonia and my nephew Deepak.

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ABBREVIATIONS

ANOVA	analysis of variance
AR	androgen receptor
ASAP	atypical small acinar proliferation
ATE	aqueous tea extracts
BPH	benign prostatic hyperplasia
C1	cell lysis buffer
C	constant
CO₂	carbon dioxide
DB	digestion buffer
DHT	dihydrotestosterone
DNA	deoxyribose nucleic acid
DRE	digital rectal examination
DU145	human brain metastatic prostate cancer cell
EGF	epidermal growth factor
EPCA-2	early prostate cancer antigen – 2
EPIC	european prospective investigation into cancer and nutrition
FAP	familial adenomatous polyposis
FCS	foetal calf serum
<i>g</i>	gravitational force
G2	general lysis buffer
hr/s	hour/s
HCl	hydrochloric acid
HGPIN	high grade prostatic intraepithelial neoplasia

HPLC	high pressure liquid chromatography
IGF	Insulin-like growth factor
<i>ig</i>	intra gavage
KD	kilodalton
LC-MS/MS	liquid chromatography-mass spectrometry/ mass spectrometry
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LNCaP	human lymph node metastatic prostate cancer cell
LPO	lipid peroxidation
M	moles per litre
mg/s	milligram/s
µg/s	microgram/s
ml/s	millilitre/s
µl/s	microlitre/s
µM	micromoles per litre
µm	micrometre
mM	millimoles per litre
M₁dG	cyclic pyrimidopurinone N-1, N ² -malondialdehyde-2'- deoxyguanosine
MDA	malondialdehyde
min/s	minute/s
nM	nanomoles per litre
MW	molecular weight

NEAA	non-essential amino acids
NSAID	non-steroidal anti-inflammatory drug
8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanosine
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + tween
PCA3	prostate cancer antigen 3
PCPT	prostate cancer prevention trial
PC3	human bone metastatic prostate cancer cell
PI	propidium iodide
PSA	prostate specific antigen
QBT	equilibration buffer
QC	wash buffer
QF	elution buffer
REDUCE	reduction by dutasteride of prostate cancer events
RNA	ribose nucleic acid
SD	standard deviation
SDS	sodium dodecyl sulphate
SELECT	selenium and vitamin E prostate cancer prevention trial
TRAMP	transgenic adenocarcinoma mouse prostate
Tris	tri-acetate-EDTA-buffer
Triton X-100	T-octylphenoxy polyoxyethanol
TRUS	transrectal ultrasound
Tween	polyoxyethene sorbitan ethylene
UV	ultra-violet

CHAPTER ONE

INTRODUCTION

1.1 Prostate Cancer

1.1.1 Incidence

Cancer of the prostate is the most commonly diagnosed solid malignancy and the second leading cause of cancer-related deaths in men living in developed countries (Metlin, 1997). It is estimated by Cancer Research UK, that in 2008 there will be over 35,000 newly diagnosed cases of prostate cancer and approximately 10,000 men will die as a result of the disease. Similarly, in North America, it is estimated that there will be 186,320 new cases and 28,660 deaths from prostate cancer in 2008 (Jemal *et al*, 2008). In the UK, the incidence is likely to double over the next 20 years due to a combination of improved longevity of the general population and more widespread use of prostate specific antigen (PSA) testing (Dutta Roy *et al*, 2005), as already noted in North America following prostate cancer screening (Rubben and Lummen, 2002; Wingo *et al*, 1997).

1.1.2 Risk factors

Although the full understanding of the aetiology of prostate cancer is unknown, a number of risk factors namely; age, family history, race and dietary factors have been implicated (Nelson *et al*, 2003).

Prostate cancer is rarely diagnosed in men younger than 50 years and increases in incidence with age. The autopsy incidence of prostate cancer is 30% of men in their 50s and 70% over the age of 80 (Muir *et al*, 1991; Pienta, 1994). However, only a small proportion will develop clinically significant prostate cancer.

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A major challenge is to identify patients who will progress to develop clinically significant disease.

Androgens play a pivotal role in the development and progression of the majority of prostate cancers (Huggins *et al*, 1994; Vailancourt *et al*, 1996). Prostate cancer never develops in men castrated before puberty (Pienta and Esper, 1993), neither is it seen in eunuchs (Wu and Gu, 1991). Indeed, it has been suggested that the increased risk of prostate cancer development and mortality among African Americans compared with Caucasian Americans may, in part, be related to chronically elevated levels of circulating androgens in the former (Ross *et al*, 1992).

Epidemiological studies show that the highest incidence of prostate cancer is amongst African Americans (Mebane *et al*, 1990), the lowest incidence being amongst South Asians, namely, Japanese men (Denis *et al*, 1999). However, observations amongst Japanese immigrants to North America show an increased incidence of prostate cancer and subsequently, generations of American born Japanese men have similar risks to those of the host population (Whitmore *et al*, 1995). These findings suggest that environmental factors play an important role in the development of prostate cancer.

Until recently, the Western diet, particularly high animal fat intake was considered to be a factor associated with increased prostate cancer risk (Statland, 1992). The results of the European Prospective Investigation into Cancer and Nutrition (EPIC) study (Crowe *et al*, 2008) have cast doubts on such an association. This multi-centre prospective study of 142,520 men found at a

Chapter 1

median follow-up of 8.7 years, that there was significant association between dietary fat intake and risk of prostate cancer. Although, the intake of soy products (isoflavonoids, phytoestrogens), lycopenes, selenium and vitamin E are associated with a reduced risk of prostate cancer development (reviewed by Pathak *et al*, 2003). Soy products feature strongly in the Japanese diet.

Mechanistically, the vast majority of the protective dietary factors are thought to exert their influence by their anti-oxidant properties (Fleshner and Kucuk, 2001).

1.1.3 Pathology

Although prostate cancer is a heterogenous, multi-focal disease (Byar and Mostofi, 1972), greater than two-thirds of the disease affects the peripheral zone of the gland (McNeal *et al*, 1988). Histologically, approximately 95% of the tumours are adenocarcinomas, the rest include transitional cell carcinomas, sarcomas and leiomyosarcomas (Cheville *et al*, 1995; Sexton *et al*, 2001).

Pre-malignant lesions

High grade prostatic intraepithelial neoplasia (HGPIN) is now widely accepted as a pre-malignant lesion for prostate cancer development (Brawer, 1992; Lipski *et al*, 1996). Recently, proliferative inflammatory atrophy (PIA) has emerged as a possible precursor lesion of HGPIN (De Marzo *et al*, 1999; Putzi and De Marzo, 2000). Atypical small acinar proliferation (ASAP) refers to the morphological features of the atypical glands but fall short of the criteria used in the histological diagnosis of prostate cancer.

High grade prostatic intraepithelial neoplasia (HGPIN)

The evidence that HGPIN is a precursor of prostate cancer is extensive (Brawer, 1992; Lipski *et al*, 1996). The prevalence of both HGPIN and prostate cancer increases with age (Sakr *et al*, 1993). According to one study, HGPIN is more frequently found in prostate glands containing cancer (83%) than in benign glands (43%) (McNeal *et al*, 1986). HGPIN is found in 10% of initial prostate biopsies (Bostwick *et al*, 2004), however, subsequent biopsies confirm a histological diagnosis of prostate cancer in 30% (Lefkowitz *et al*, 2002). HGPIN is a histological lesion that shares genetic and cellular similarities with prostate cancer but without invasion of the basement membrane (Sakr *et al*, 1993; Sakr *et al*, 2001). Cytological similarities include variability in nuclear size, enlargement of nuclei and prominent nucleoli (Montironi *et al*, 2007). Anatomically, HGPIN is frequently found in the peripheral zone of the prostate gland, the site at which 70% of prostate carcinomas occur (Bostwick and Brawer, 1987; Sakr *et al*, 1993). It has been suggested that HGPIN may take 20-30 years to develop into prostate cancer, and progression to clinically significant cancer may take between 3-15 years (Bostwick, 1992). HGPIN is potentially a reversible pre-malignant condition. Vailancourt *et al*. (1996) showed that men with histological evidence of prostate cancer and HGPIN were found to have a significant reduction in prostate cancer and HGPIN on repeat prostate biopsies following three months of androgen deprivation therapy. Monitoring HGPIN with serial prostate biopsies is fraught with sampling error and thus, it is extremely difficult to determine the true reversibility of HGPIN. Therefore, one must exercise caution when describing the reversibility of HGPIN and thus, it should be regarded as a potentially rather

than truly reversible lesion. Coupled with a long natural history and the potential reversibility of this lesion, considerable scope exists for the chemoprevention of prostate cancer (Alberts *et al*, 2006; Cheng *et al*, 2004).

Proliferative inflammatory atrophy (PIA)

PIA is a lesion that is recognised by groups of highly proliferative prostatic epithelial cells intermingled with inflammatory cells and focal atrophy (De Marzo *et al*, 1999). It has been hypothesized that PIA is a pre-malignant condition similar to the pre-malignant inflammation observed in liver and large bowel (Ruska *et al*, 1998). Recently, it has been suggested that PIA may be a precursor lesion of HGPIN and/or directly progresses to prostate cancer (Putzi and De Marzo, 2000). Therefore, this lesion may also be a target for chemoprevention of prostate cancer. Currently, targeting HGPIN and PIA as intermediate surrogate biomarkers for chemoprevention studies is fraught with sampling errors of both primary and subsequent prostate biopsies (Bono *et al*, 2007).

Atypical small acinar proliferation (ASAP)

ASAP was originally described by Iczkowski (Iczkowski *et al*, 1997). It refers to atypical glands that strongly resemble the morphological characteristics of prostate cancer, however, fall short of the histological features for cancer diagnosis. The prevalence of ASAP on initial prostate biopsies is estimated to be 1.5–5%, with the confirmed diagnosis of prostate cancer in up to 60% of repeat biopsies (Epstein and Potter, 2001).

1.1.4 Natural history

The development and progression of prostate cancer is considered to be a multi-step process (Figure 1.1.4.1). The timeframe of the natural history of the disease is thought to be a several decades (Bostwick, 1992). Initially, the pre-malignant lesion, HGPIN develops over 20-30 years. Subsequently, the development of clinically significant prostate cancer may take 3-15 years (Bostwick, 1992). During this period, localized and metastatic cancer may develop. All early prostate cancers are considered to be androgen-dependent. In fact, about 75% of metastases are also androgen-dependent (Wilding, 1995), subsequently; androgen-independence is achieved. A variety of molecular mechanisms are implicated in this process. Firstly, the promiscuity of the androgen receptor for ligands other than androgens is widely recognised. Molecules, such as insulin-like growth factor (IGF) and epidermal growth factor (EGF) can increase androgen receptor transcriptional activity in the absence of androgens (Culig *et al*, 1994) (Figure 1.1.4.2). Secondly, with castrate levels of androgens, the androgen receptor pathway can become up regulated by gene amplification (Linja *et al*, 2001). Finally, point mutations within the androgen receptor gene may lead to androgen independence (Taplin *et al*, 1995).

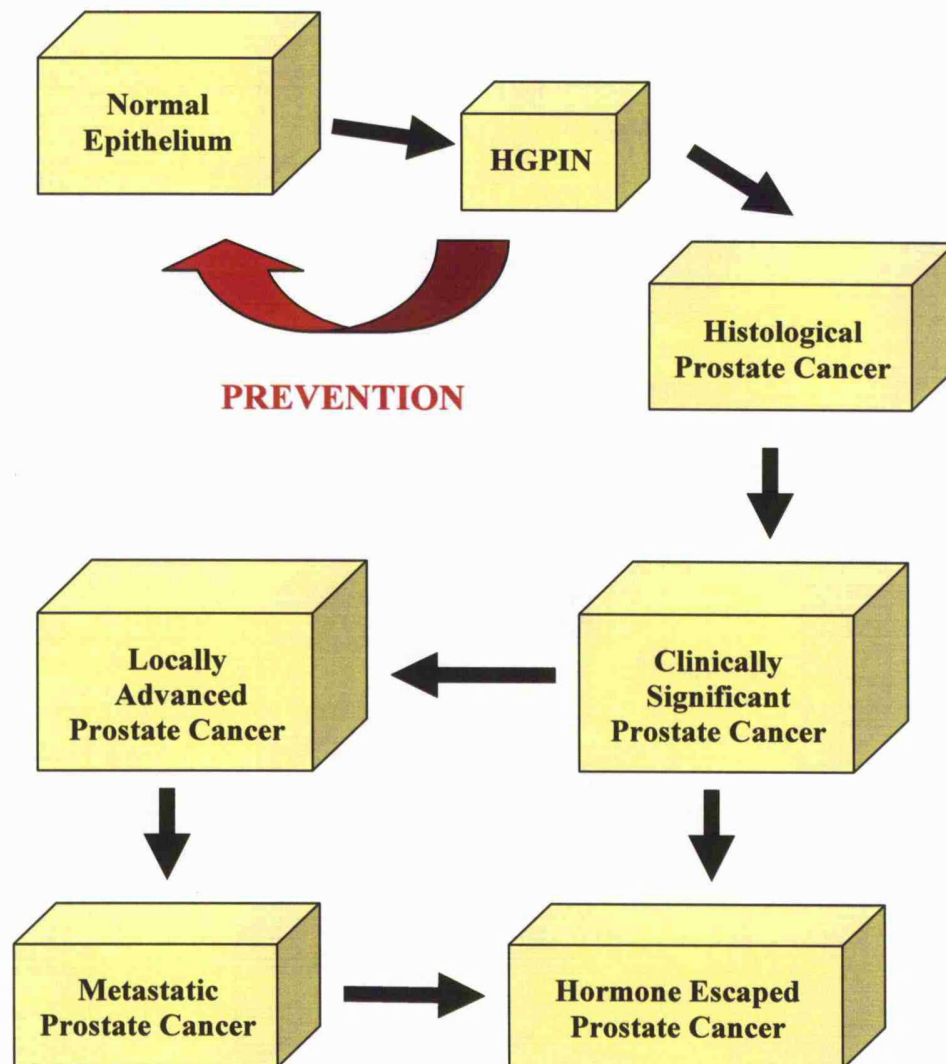


Figure 1.1.4.1 Theoretical multi-step model of prostate carcinogenesis and progression. The long latency period of clinically significant prostate cancer development provides an ideal opportunity for prevention strategies. This may be aimed at HGPIN (high grade prostatic intraepithelial neoplasia) lesions. Failure to manage clinically localised prostate cancer may then ultimately result in metastatic disease and hormone escaped prostate cancer (Figure modified from Lamb and Zhang 2005).

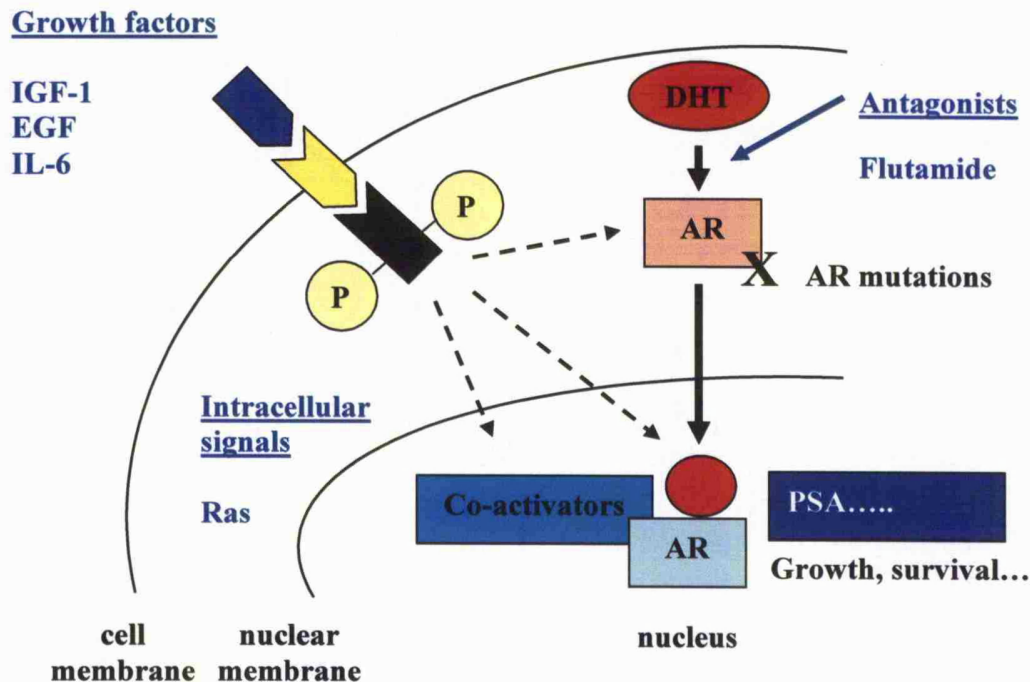


Figure 1.1.4.2 Mechanisms of ligand-independent androgen receptor activation. Dihydrotestosterone (DHT) is converted from testosterone by the enzyme 5 α -reductase chiefly found in stromal cells. The DHT leaves the stromal cells and binds to the androgen receptor (AR) in the cytoplasm of prostatic epithelia. Subsequently, this DHT-AR complex moves into the cell nucleus and binds to DNA, resulting in the activation of regulatory genes of cell division and growth. This pathway is inactivated when there are mutations in the AR gene. However, the AR receptor may still function by activation independent of the DHT-AR complex. Such independent growth factors include insulin-like growth factor (IGF) and epidermal growth factor (EGF).

1.1.5 Diagnosis of prostate cancer

A combination of clinical examination of the prostate gland, serum prostate specific antigen (PSA) testing and transrectal ultrasound guided biopsy (TRUS) remain the current methods employed to diagnose prostate cancer. Ultimately, the definitive diagnosis of prostate cancer is confirmed by histological examination of prostatic tissue.

Digital rectal examination and TRUS

Digital rectal examination (DRE) forms the initial clinical step in the diagnosis of prostate cancer. Over 20 years ago, prior to widespread PSA testing, prostate cancer was usually detected by DRE, by which time the cancer had often spread (Thompson *et al*, 1984). With increasing PSA testing, detection of prostate cancer is now commonly diagnosed in its early stages, therefore, DRE is often found to be normal (Derweesh *et al*, 2004).

As mentioned above, prostate cancer is considered to be a multi-focal disease of the prostate gland. Prostate biopsies are, therefore, taken in a random systematic manner from within the gland under TRUS guidance (Hodge *et al*, 1989; Ravery *et al*, 1999). The classical hypoechoic lesions seen on ultrasound only correlate with 30% of prostate cancer diagnosis on subsequent biopsies of these lesions (Ellis *et al*, 1994). The presence of HGPIN and ASAP in the specimen greatly increases cancer detection of subsequent biopsies by 20% and 60%, respectively (Herawi *et al*, 2006; Lefkowitz *et al*, 2002).

Prostate Specific Antigen

PSA is a 33 kilodalton (KD) serine protease that is secreted by the epithelium of the prostate (Wang *et al*, 1979). The primary function of PSA is to liquefy the seminal coagulum (Lilja and Laurell, 1984). PSA is secreted in high concentrations (mg/ml) into seminal fluid (McGee and Herr, 1988), however, it is normally found in low concentrations in serum (ng/ml) (Papsidero *et al*, 1980). Despite PSA being organ-specific and not cancer-specific, since its introduction, PSA has revolutionised the diagnosis of prostate cancer (Polascik, *et al*, 1999). In addition to prostate cancer, serum PSA levels are elevated in non-cancerous prostate conditions such as in benign prostatic hyperplasia (BPH), prostatitis, retention of urine and prostate gland biopsies (Armitage *et al*, 1988; Stamey *et al*, 1987). Until recently, the upper limit of normal for serum PSA was considered to be 4 ng/ml. Prostate cancer detection rates in screened populations are 27% and 59% for PSA levels of 4.1-9.9 and ≥ 10 ng/ml, respectively (Catalona *et al*, 1993). PSA density (Benson *et al*, 1992), PSA density of the transitional zone (Zlotta *et al*, 1997), age-specific range PSA (Oesterling *et al*, 1993) and PSA velocity (Carter *et al*, 1992) have all been used to improve its specificity in prostate cancer diagnosis. However, despite the limitations in diagnosis, it is still a very useful test in monitoring prostate cancer therapy (Pound *et al*, 1999).

Therefore, although PSA has been used as a screening tool, the limitations have stimulated intense interest in the search for alternative biomarkers.

1.1.6 Androgen regulation

Biosynthesis of androgens

Testosterone is the principal male androgen. It is synthesised from cholesterol in the Leydig cells of the testes, accounting for 95% of overall testosterone production. The remaining 5% is synthesised from the precursor, androstenedione in the adrenal gland. The synthesis is under the influence of the hypothalamic-pituitary-gonadal axis. Luteinizing hormone-releasing hormone (LHRH) produced by the hypothalamus stimulates the pituitary gland to secrete luteinizing hormone (LH), which stimulates the Leydig cells in the testes to produce testosterone. The homeostasis of testosterone is maintained by a negative feedback mechanism at both the hypothalamic and pituitary level (Figure 1.1.6.1).

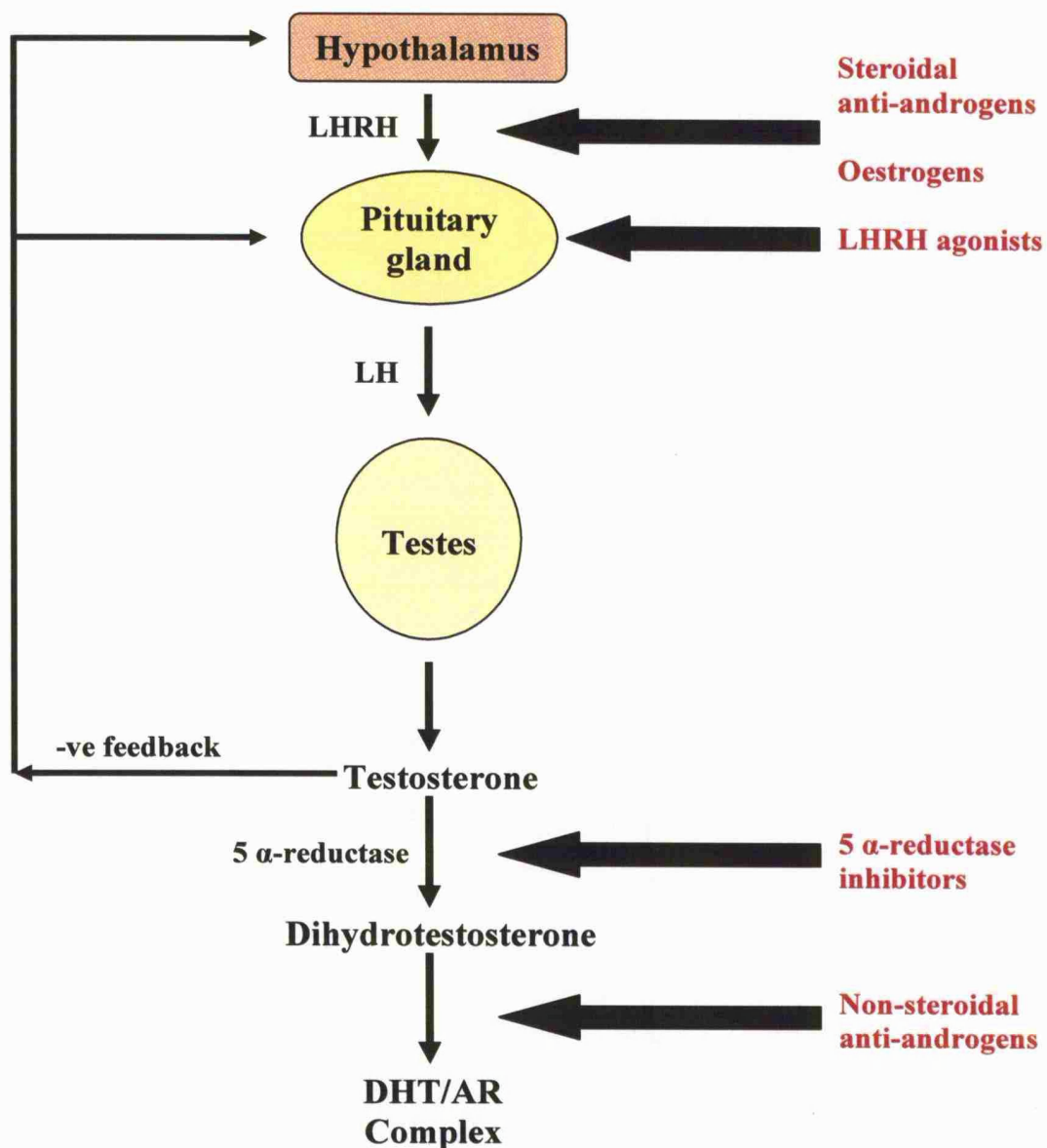


Figure 1.1.6.1 Androgen homeostasis at the hypothalamic-pituitary-gonadal axis.

The homeostasis of serum testosterone and dihydrotestosterone (DHT) is chiefly governed by the hypothalamic-pituitary-gonadal axis. The phasic secretion of Luteinizing Hormone-Releasing Hormone (LHRH) from the hypothalamus stimulates the pituitary gland to secrete Luteinizing Hormone (LH). Leydig cells of the testes are stimulated by LH to produce and secrete testosterone. These pathways may be manipulated in prostate cancer by reducing or blocking the effects of androgens (shown in red).

Mechanism of action

Like other steroid hormones, testosterone binds to a receptor within the cytoplasm; this steroid-receptor complex then passes into the cell nucleus and binds to DNA, facilitating transcription of a number of androgen-dependent genes involved in cell division and proliferation (Steers, 2001). In some target tissues including prostate epithelium, testosterone under the influence of the enzyme 5 α -reductase is converted to dihydrotestosterone (DHT). DHT is the most potent androgen (Wright *et al*, 1996) (Figure 1.1.6.2). There are 2 isoenzymes of 5 α -reductase, type I and II. Type I 5 α -reductase is expressed primarily in the skin and liver and to a lesser extent in the prostate gland; type II is expressed primarily in the prostate gland (Kokontis and Liao, 1999).

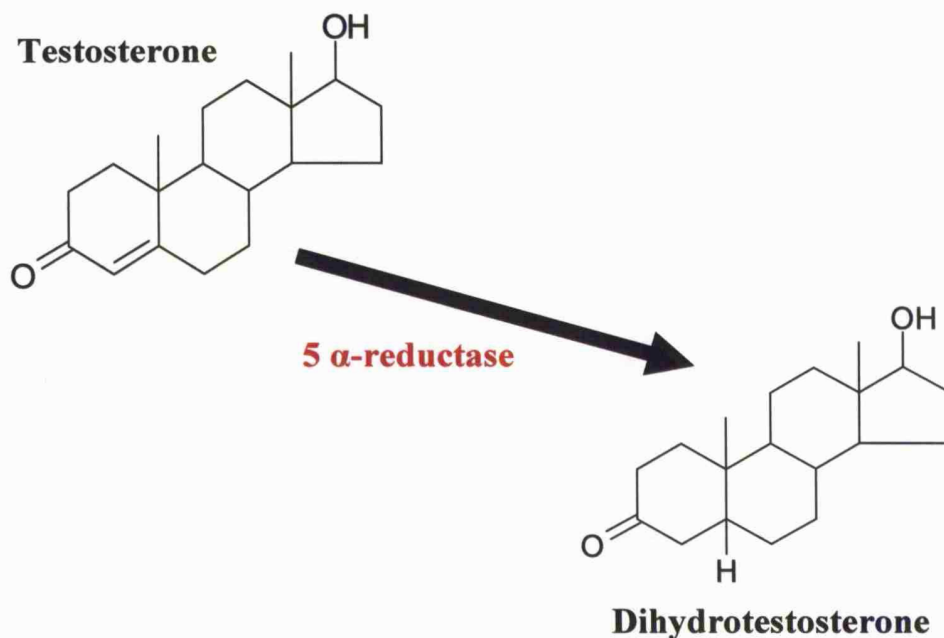


Figure 1.1.6.2 Chemical structures of Testosterone and Dihydrotestosterone. (288 MW and 290 MW, respectively).

1.1.7 Prostate cancer treatment

Over the past decade, increased PSA testing has led to an increased detection rate of organ-confined prostate cancer (Jhaveri *et al*, 1999). Therefore, curative treatment of prostate cancer; namely; surgery and radiotherapy have been the preferred methods used, replacing the traditional anti-androgen therapy. That said, anti-androgen therapy may still be used in the adjuvant or neoadjuvant setting. Locally advanced and metastatic cancers are primarily still treated with anti-androgens.

Historical perspectives

The beneficial effects of androgen ablation in prostate cancer were not realized until 1941, when Huggins and Hodges treated patients suffering with metastatic prostate cancer with surgical castration or oestrogen therapy, resulting in patients gaining weight, appetite and most notably experiencing reduced bone pain (Huggins *et al*, 1941). Later, medical castration with oral oestrogens resulted in prostate cancer becoming the first cancer to be systemically treated. To this day, androgen ablation therapy remains the mainstay for locally advanced and metastatic prostate cancer.

Androgen blockade

Surgical castration

Following the work of Huggins in 1941, surgical castration became the accepted practice for advanced prostate cancer. In the 1960s, the veterans administration cooperative urological research group (VACURG) carried out a randomised trial comparing the effects of oral administration of oestrogens versus orchidectomy in

prostate cancer patients. They concluded that oestrogen therapy was as effective as orchidectomy (Byar and Corle, 1988). Later it became evident that oestrogen therapy resulted in cardiovascular and thrombo-embolic complications. It also became evident that oestrogen therapy or orchidectomy was not sufficient to completely cure some patients of the disease; in these patients adrenal glands production of androgens was sufficient to continue stimulating the growth of prostate cancer (Huggins and Scott, 1945).

Medical castration

Anti-androgens inhibit androgen action by competitively binding to the AR. They can be classified as steroidal and non-steroidal. Cyproterone acetate is a steroidal anti-androgen; centrally, it blocks the release of LH and at a cellular level blocks the action of androgens (Jacobi *et al*, 1980). The resulting reduced serum testosterone produces a loss of libido and erectile dysfunction. Flutamide is a non-steroidal anti-androgen (Figure 1.1.7.1) which inhibits both testosterone and DHT from binding to the AR (Labrie, 1993; Neri *et al*, 1967). Therefore, serum testosterone levels do not decrease as seen with steroidal anti-androgens. In some cases, continuation of flutamide therapy, paradoxically; stimulates prostate cancer growth, stopping flutamide leads to regression of prostate cancer, this phenomenon is known as “anti-androgen withdrawal” (Taplin *et al*, 1995).

The anti-androgens have largely been replaced with LHRH agonists; given that the latter can be administered subcutaneously, once every three months. The reduction of serum androgens to similar levels found in castrated men is produced by LHRH agonists (Vilchez-Martinez *et al*, 1979).

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Mechanistically, at the initiation of LHRH therapy, there is a surge in LH and testosterone levels. The increased levels of LH and testosterone lead to negative feedback inhibition at the hypothalamus and pituitary gland, resulting in the loss of phasic LH release. In the absence of LH, Leydig cell production of testosterone drops to castrate levels (Figure 1.1.6.1).

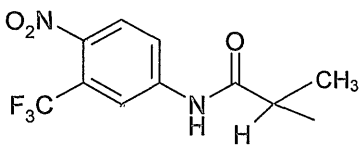


Figure 1.1.7.1 Chemical structure of Flutamide (276 MW).

1.2 Chemoprevention

1.2.1 General concepts

Cancer chemoprevention is the administration of agents (drugs, biologics and nutrients) to prevent induction or inhibit or delay the progression of cancers (Sporn and Suh, 2000). The development of chemopreventive strategies requires knowledge of the mechanisms of carcinogenesis and identification of agents that interfere with these mechanisms. Currently, the large cohort size necessary and the prolonged length of chemoprevention trials signify a need for identifying and characterising early intermediate biomarkers. The validation of such markers as surrogate endpoints for cancer incidence is essential for chemopreventive agent development (Trock, 2001). Suitable populations with appropriate risk factors, including the presence of pre-malignant lesions and genetic predispositions need to be well characterized for future chemopreventive interventions.

1.2.2 Chemoprevention trials in prostate cancer

Rationale

The particularly long latency period of prostate carcinogenesis along with the potential reversibility of the pre-malignant lesion, HGPIN, provides an ideal window of opportunity for chemoprevention using drugs or diet-derived agents. No chemopreventive agent can be considered of proven value unless it has been tested in randomised controlled trials. At present these trials are of long duration and extremely expensive. One of the reasons is that the principal endpoints used in Phase III trials is prostate cancer incidence. The development of biomarkers of efficacy of intervention as well as of prostate carcinogenesis *per se* should encourage the advancement of agents to the Phase I-II clinical trial level. Assays

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for such biomarkers need to be of high specificity, sensitivity, reproducibility and practical for the analysis of large numbers of clinical samples (Rothman *et al*, 1995). Currently, HGPIN is under intense genetic and molecular scrutiny as a histological stage that provides an opportunity for validating biomarkers of prostate carcinogenesis (Kelloff *et al*, 2000). The study into the role of oxidative species in prostate cancer may accelerate over the next decade (Fleshner and Kucuk, 2001). Particularly, with the development of assays measuring cellular DNA, proteins and lipids damage (Haegle *et al*, 1994).

The concept that oxidative stress may play an important role in the development and progression of prostate cancer is relatively new. The impetus for experimental and clinical research into the role of oxidative stress and prostate cancer has greatly increased following important findings in 2 clinical trials. The Alpha-Tocopherol and Beta-Carotene study (ATBC study) was a double blind randomised, placebo-controlled trial of 29,133 male smokers receiving two anti-oxidants; alpha-tocopherol, beta-carotene, alpha-tocopherol and beta-carotene in combination, or a placebo. Lung cancer incidence was the principal endpoint measured. Paradoxically, the trial showed an increased incidence of lung cancer amongst the subgroup of men who received beta-carotene supplements (The ATBC Cancer Prevention Study Group. 1994). However, secondary analysis of data at six years of follow-up, demonstrated a 32% reduction in the incidence and a 41% reduction in mortality from prostate cancer among men who received supplementary alpha-tocopherol (Heinonen *et al*, 1998). The non-melanoma skin cancer prevention trial was the second trial (Clark *et al*, 1996). In this trial, 1,312 patients were randomised into two groups receiving either selenium supplements

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or placebo. The study did not show a protective effect on skin cancer incidence, however, secondary analysis at follow-up showed a 60% reduction in the incidence of prostate cancer. The antioxidant properties exerted by selenium and α -tocopherol (vitamin E) in quenching reactive oxygen species (ROS) are their principal mechanisms of action (Redman *et al.*, 1998; Shklar and Oh, 2000). There is a number of chemoprevention trials in prostate cancer underway or completed (Table 1.2.2.1).

Prostate Cancer Prevention Trial

The Prostate Cancer Prevention Trial (PCPT) was stopped approximately 15 months early by the data and safety monitoring committee who concluded that the primary endpoint of a 25% risk reduction in the treatment arm had been reached. Furthermore, sensitivity analyses suggested that additional follow-up would not change the outcome. This large randomised, placebo controlled-trial was essentially based on the concept that androgens play a pivotal role in prostate carcinogenesis.

The trial began in 1993, recruited 18,882 healthy male volunteers, aged 55 years and above with a normal DRE of the prostate gland and a serum PSA < 3.0 ng/ml. Men in the treatment group received the type II 5-alpha reductase inhibitor, finasteride daily (5 mg) and participants in the control arm of the trial received the placebo. All men were screened annually by serum PSA levels and DRE. The primary endpoint was the incidence of prostate cancer during the 7 years of the trial, as diagnosed by either for-cause biopsy (abnormal PSA or DRE) or by end-of-study biopsy. The results found a relative risk reduction of prostate cancer of

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24.8% in the finasteride arm. However, despite the reduced risk of cancer, this arm of the study was found to have a higher proportion of high grade tumours (6.4%) compared to the control arm (5.1%). This latter finding has generated considerable debate as to whether finasteride should be used as a chemoprevention agent for prostate cancer (Thompson *et al*, 2003).

Cohen *et al.* (2007) hypothesised that the increase in high grade cancers in men of the finasteride arm compared with the placebo arm of the trial, were due greater detection than true prevalence. They found that prostate size in the finasteride group was 25% smaller than in the placebo group. Therefore, when the prostate size was taken into consideration, there was not a statistically significant difference between the prevalence of high-grade prostate cancer in the two groups. Recently, the effect of bias on high-grade tumour detection was addressed in extrapolation of the rates of high-grade tumours in men who underwent radical prostatectomies (~25% of the total number of men who had biopsy-detected cancer) to the entire population of men with prostate cancer in the PCPT (Redman *et al*, 2008). This analysis showed that the true rate of high-grade prostate cancer in the finasteride-treated men (6.0%) was 27% lower than the rate in the placebo-treated men (8.2%). Thus, Redman and colleagues were able to conclude that there was no statistically significant increase in high-grade cancer, in contrast to the previously reported result. The findings by Pinsky *et al.* (2008) also support the conclusions of Redman *et al.* (2008).

Table 1.2.2.1 Prostate cancer chemoprevention trials currently sponsored by the US National Cancer Institute. Clinical trials shown are either recruiting patients or have fully recruited at the time of publication of this thesis. ECOG, Eastern Cooperative Oncology Group. SWOG, Southwest Oncology Group. Information obtained from the website of the NCI website (<http://www.nci.nih.gov/>).

Phase	Agent(s) / Drug(s) under investigation	Principal Investigator(s)
I	Celecoxib	M. Carducci (Maryland, USA)
I	Vitamin D	E. Giovannucci (Boston USA)
I	Lycopene	D. Gustin (Illinois, USA)
II	Soy	A. Venook (California, USA) I. Berkowitz (Delaware, USA) G. Fleming (Illinois, USA) D. Hurd (North Carolina, USA)
II	Polyphenon E	N. Kumar (Florida, USA)
II	Doxercalciferol	M. Cohen (Iowa, USA) W. See (Wisconsin, USA) G. Wilding (Wisconsin, USA)
II	Flutamide and Toremifene	J. Nelson (Pennsylvania, USA)
II	Fish oil and Green Tea	J. Shannon (Oregon, USA)
III	Selenium	D. Jarrard (ECOG, USA) J. Marshal (SWOG, USA)
III	Selenium / Vitamin E	D. Karp (ECOG, USA) E. Klein (SWOG, USA)

Reduction by dutasteride of prostate cancer events trial

The reduction by dutasteride of prostate cancer events (REDUCE) trial was designed and has now completed recruitment of 8,000 men. The rationale for this trial was based on a number of flaws of the PCPT trial. Firstly, finasteride is a type II 5 α -reductase inhibitor which is found to be the predominant isoenzyme in normal and benign prostatic tissues. However, dutasteride is both a type I and type II 5 α -reductase inhibitor. The type I isoenzyme is found to be expressed in greater amounts in prostate cancer cells than normal prostate cells (Bruchovsky *et al*, 1996; Thigpen *et al*, 1993; Thomas *et al*, 2003). Secondly, strict eligibility criteria for the REDUCE trial were set. These include;

- Men between 50-75 years
- Serum PSA between 2.5 -10 ng/ml (aged 50-60 years)
- Serum PSA between 3.0 -10 ng/ml (aged > 60 years)
- No histological evidence of prostate cancer on biopsy
- No evidence of HGPIN
- Prostate size <80 cm³

REDUCE is a 4 year international, multi-centre, randomised, double blind placebo-controlled trial. Each arm received either dutasteride (0.5mg) or placebo daily for the 4 year period. The primary endpoint is biopsy-determined prostate cancer at 2 and 4 years of treatment. Secondary endpoints will incorporate genetic and protein biomarkers (Andriole *et al*, 2004).

Selenium and Vitamin E Cancer Prevention Trial

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) has fully recruited men under the co-ordination of the South West Oncology Group (SWOG), San Antonio, Texas. This double blind randomised, placebo-controlled trial has recruited 32,400 men, who will receive daily supplements of a combination of two of the following: Selenium (200 micrograms), vitamin E (400 mg) or placebo. The study is expected to take 12 years to yield meaningful data regarding the significance of vitamin E, selenium and the combination of the two in preventing prostate cancer. The principal endpoint of the trial is the incidence of prostate cancer, assessed by routine clinical investigation including annual DRE and serum PSA measurements. In addition, quality of life will be assessed. Blood and prostate tissue samples will be stored for subsequent analyses of micronutrient levels and certain biological and genetic biomarkers of prostate cancer. The incidence of lung and colorectal cancers will also be studied (Klein *et al*, 2000).

The long timeframe projected for the SELECT demonstrates an important issue in the advancement of potential chemopreventive agents from preclinical to large-scale clinical trials. As mentioned above, if the incidence of cancer acts as the only endpoint measured in chemoprevention trials, prolonged time periods will be necessary to obtain results. More importantly, during this prolonged period of time, no indication will be available on the potential efficacy of the intervention, nor regarding harm or benefit caused to participants. Furthermore, the PCPT trial has highlighted significant doubt of having a normal cut-off limit for serum PSA.

In the control arm of the PCPT, 2,950 men with a serum PSA <4.0 ng/ml, 449 had a histological diagnosis of prostate cancer (Table 1.2.2.2). Therefore, there is an intense need to discover suitable biomarkers for the chemoprevention of prostate cancer.

PSA (ng/ml)	Number of men	Number of men with prostate cancer (%)
≤ 0.5	486	32 (6.6)
0.6 – 1.0	791	80 (10.1)
1.1 – 2.0	998	170 (17)
2.1 – 3.0	482	115 (23.9)
3.1 – 4.0	193	52 (26.9)

Table 1.2.2.2 Relationship between low serum PSA level and the prevalence of prostate cancer. Adapted from Thompson *et al.* (2004).

1.2.3 Biomarkers

At present chemoprevention trials are large and of long duration, therefore, expensive. There is a need to develop biomarkers that can be used to assess progress throughout the time course of such trials. Such biomarkers would assess intermediate endpoints, reflect biological events that take place between exposure to external or endogenous carcinogens and the subsequent development of cancer (Kelloff *et al*, 1994). They may also be referred to as “biomarkers of carcinogenesis”.

HGPIN and serum PSA are two potential biomarkers that may be used in chemoprevention trials for prostate cancer but neither has been completely validated. HGPIN has limitations due to the technical problems associated with tissue sampling and repeat biopsies (Bono *et al*, 2007). Although PSA is a good marker to assess therapeutic efficacy (Pound *et al*, 1999), its use as a biomarker for chemoprevention in prostate cancer has been difficult to demonstrate, for example, serum PSA is not affected in cases of HGPIN (Ronnett *et al*, 1993).

Common properties shared by ideal biomarkers of early carcinogenesis are (Einspahr *et al*, 1997);

- Variability of expression between phases of the carcinogenesis pathway.
- Ability to be detected early along the carcinogenesis pathway.
- Association between level of biomarker and the risk of cancer development.
- Presence in tissues which are easily accessible for biopsy.

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- Potential for modification by a chemopreventive agent.
- Reproducibility in laboratory assays.

In selecting biomarkers, it is important to consider the ease and safety within which they can be assayed. Tissue markers will require frequent biopsies, which in the case of prostate tissue carry the risks and complications as discussed earlier. Therefore, biomarkers assayed from blood or urine that reflects changes in target tissue would be ideal.

Identification of suitable cohorts

Five target populations appear to have the greatest potential for chemopreventive trials in prostate cancer.

1. Patients with HGPIN.
2. Patients with an early cancer scheduled to be treated by active monitoring.
3. Patients with cancer scheduled for a radical prostatectomy 6 to 8 weeks following diagnosis.
4. High risk patients with elevated PSA values or a family history of early onset prostate cancer that undergo prostate biopsies upon study entry.
5. Normal men (PSA < 3.0 ng/ml, normal DRE) from the general population but with some increased risk factors (increased age, racial group etc).

One of the great challenges for the progress of prostate cancer chemoprevention trials is the development and validation of biomarkers. Currently, the biomarkers showing greatest potential in the diagnosis of prostate cancer are the early prostate cancer antigen-2 (EPCA-2) and prostate cancer antigen 3 (PCA3).

EPCA-2

Initially, EPCA antibodies were shown to have increased staining in prostate cancer tissue compared with controls (Dhir *et al*, 2004). Subsequently, this led to the development of a serum immunoassay for detecting EPCA. EPCA was identified in 92% of patients with prostate cancer (Paul *et al*, 2005). Using a cut-off value of 1.7 absorbance at 450 nm, only men with prostate cancer expressed plasma EPCA-2 levels. Clearly, a larger trial is needed but great potential exists using EPCA-2 to identify men suffering from prostate cancer and thus, who may benefit from prostate biopsies.

PCA3

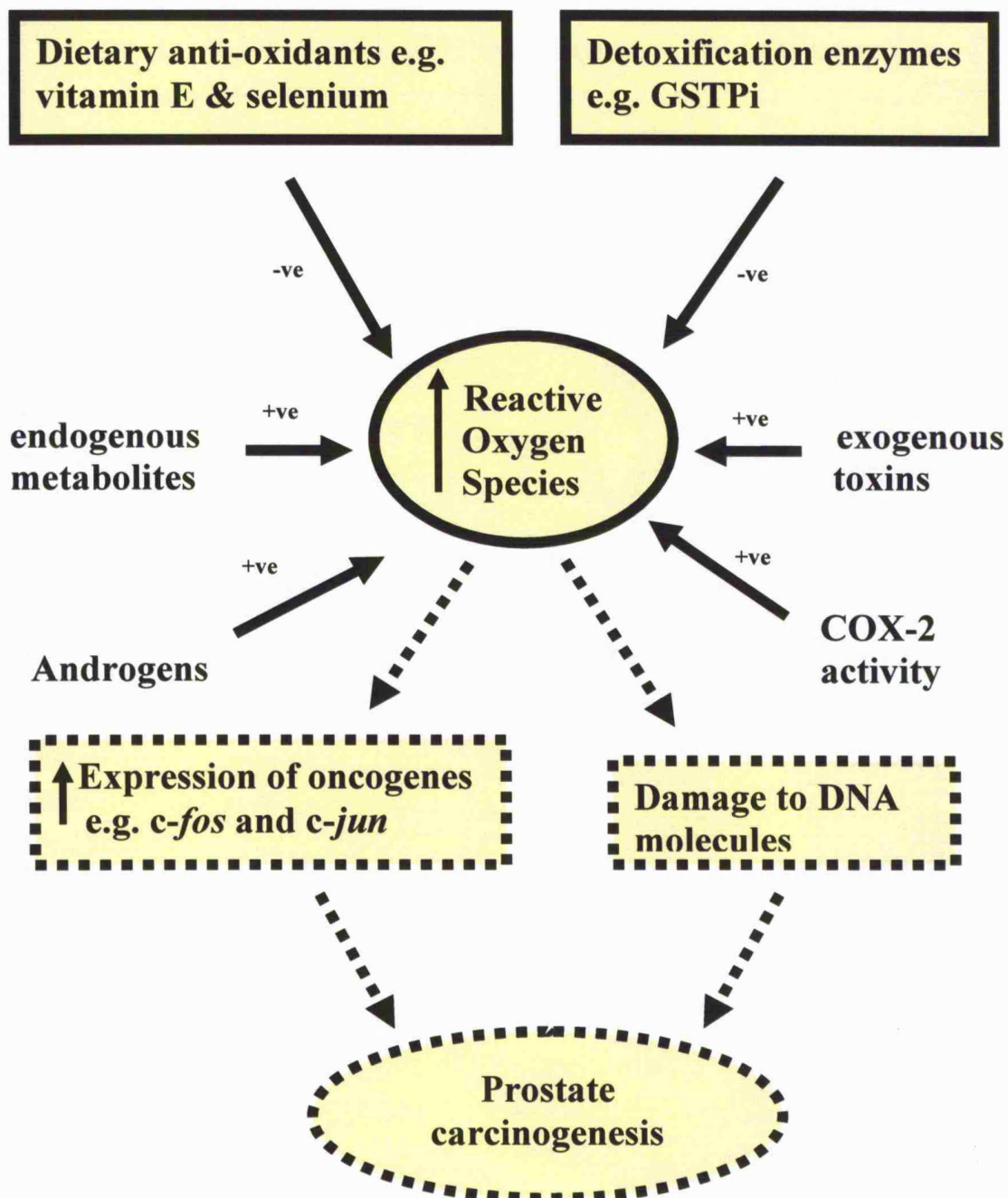
PCA3 is a non-coding RNA that is very specific to prostate epithelium (Bussemakers *et al*, 1999). It is highly expressed in both prostate cancer specimens and prostate cancer metastasis with more than 60-fold upregulation over benign tissue (Hessels *et al*, 2003). Following prostatic massage, PCA3 mRNA levels can be measured in urinary sediment. Despite there being a number of diagnostic tests, only one is commercially available (Groskopf *et al*, 2006). This test kit, APTIMA[®], uses transcription mediated amplification. A recent multi-institutional study of 534 men undergoing prostate biopsies for suspected cancer compared the PCA3 scores with serum PSA levels (van Gils *et al*, 2007). The results showed a significant correlation of PCA3 scores with prostate cancer detection which outperformed PSA. Furthermore, in men with prostate cancer who had undergone an initial negative biopsy PCA3 was shown to have greater sensitivity and specificity for prostate cancer detection than PSA (Marks *et al*, 2007).

EPCA-2 and PCA3 have potential to supercede PSA as biomarkers for prostate cancer. These molecular biomarkers will require reproducibility and standardisation of assays to progress further (Wright and Lange, 2007).

1.3 Oxidative stress and oxidative DNA damage

Normal cellular processes, inflammation and environmental factors such as diet and ionizing radiation may contribute to the generation of ROS within cells. Oxidative stress is defined as a disturbance in the equilibrium between ROS and detoxifying anti-oxidant systems. An excess of ROS leads to oxidative damage to cellular constituents (Figure 1.3.1). The balance is normally maintained by two principal mechanisms which render ROS less harmful. Firstly, intracellular anti-oxidants, such as selenium, vitamins C and E quench ROS. Secondly, anti-oxidant enzymes, such as glutathione *S*-transferase- π (GST- π) catalyse the conjugation of ROS to glutathione rendering them less harmful. Common ROS of biological significance include hydrogen peroxide, superoxide anions and hydroxyl radicals. Sources of ROS can be subdivided into endogenous and exogenous, both types causing oxidative damage to important cellular molecules including lipids, proteins and DNA (Fleshner and Klotz, 1998). ROS may react with DNA bases to produce oxidative DNA adducts. Such adducts have been associated with both mutagenesis and carcinogenesis (Marnett, 2000). ROS may directly damage DNA leading to the formation of the DNA adduct 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxo-dG) (Figure1.3.2).

Figure 1.3.1 Homeostasis of reactive oxygen species (ROS) metabolism: the potential for ROS to induce prostate carcinogenesis. Key: increase in ROS production (+ve), decrease in ROS production (-ve), malignant transformation pathway (▣➔).



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Indirect mutagenic actions of ROS include lipid peroxidation of cellular membranes, resulting in the production of compounds, such as malondialdehyde (MDA) which are capable of reacting with DNA bases resulting in the formation of adducts. The reaction of MDA with DNA results in the formation of cyclic pyrimidopurine N-1, N² malondialdehyde-2'-deoxyguanosine adduct, M₁dG (Marnett, 2000) (Figure 1.3.3). ROS can also increase the expression of transcriptional factors including *c-fos* and *c-jun* proteins involved in oncogenic progression (Toledano and Leonard, 1991; Wasylyk and Wasylyk, 1993; Wei, 1992).

It has been shown that oxidative stress can be measured in prostate cancer cells (O'Ripple *et al*, 1997; Sun *et al*, 2001), and that such species are strongly linked with oxidative damage (Malins *et al*, 1997). A pro-oxidant state, which can be achieved by either an increase in ROS or a disruption of the normally protective anti-oxidant systems, has been observed in both HGPIN and prostate cancer (Lee *et al*, 1994).

Oxidative DNA adduct: 8-oxo-dG

8-oxo-dG has been the most commonly measured oxidative DNA adduct in humans (Floyd *et al*, 1986). *In vitro*, it has been associated with mutagenesis in bacterial and mammalian cells. It also induces G:C and T:A transversions after replication in human DNA, mutations seen frequently in oncogenes and tumour suppressor genes (Cheng *et al*, 1992; Moriya, 1993; Shibutani *et al*, 1991). Levels of 8-oxo-dG have been measured in prostate tissues. The daily administration of selenium (strong anti-oxidant) to aged canines for 7 months, revealed a reduction in oxidative DNA compared with controls. This was reflected by a reduction of 8-oxo-dG levels in prostate tissue and blood leucocytes (Waters *et al*, 2003). Clinically, increased levels of 8-oxo-dG have been found in human BPH compared to adjacent normal prostate tissue (Olinski *et al*, 1995). A study of normal and malignant human prostate tissues revealed a highly significant increase in the proportion of mutagenic base lesions with age, with intersection of the two slopes at 61 years, an age at which prostate cancer is known to commonly occur (Malins *et al*, 2001). A phase II trial performed in men undergoing radical prostatectomies for prostate cancer, who received supplements of tomato sauce (containing high levels of the anti-oxidant, lycopene) for 3 weeks prior to surgery, pre- and post-treatment levels of 8-oxo-dG were measured (Chen *et al*, 2001). Post-treatment levels of 8-oxo-dG were significantly reduced in both prostatic tissue and blood leucocytes. This trial indicates that measurable levels of oxidative stress are present in established neoplasia of the prostate and blood leucocytes; therefore, 8-oxo-dG may act as biomarker influenced by dietary intervention over a short timeframe.

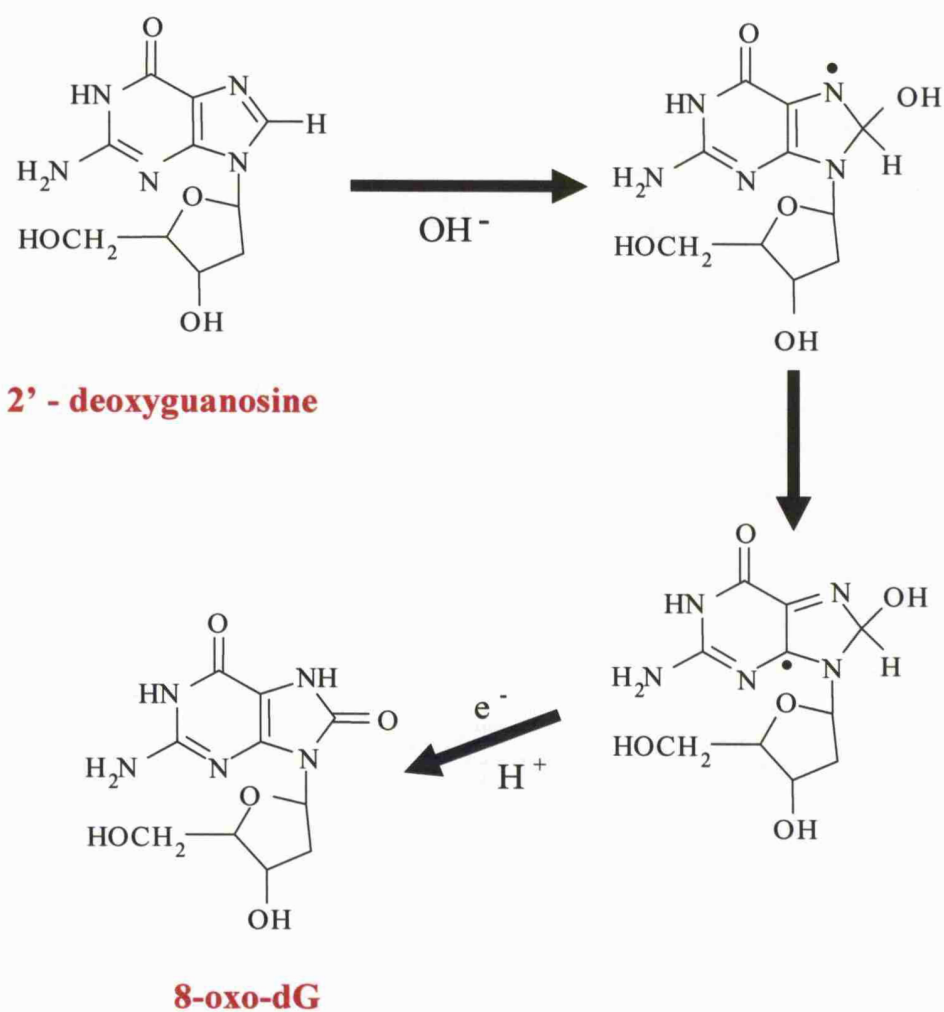
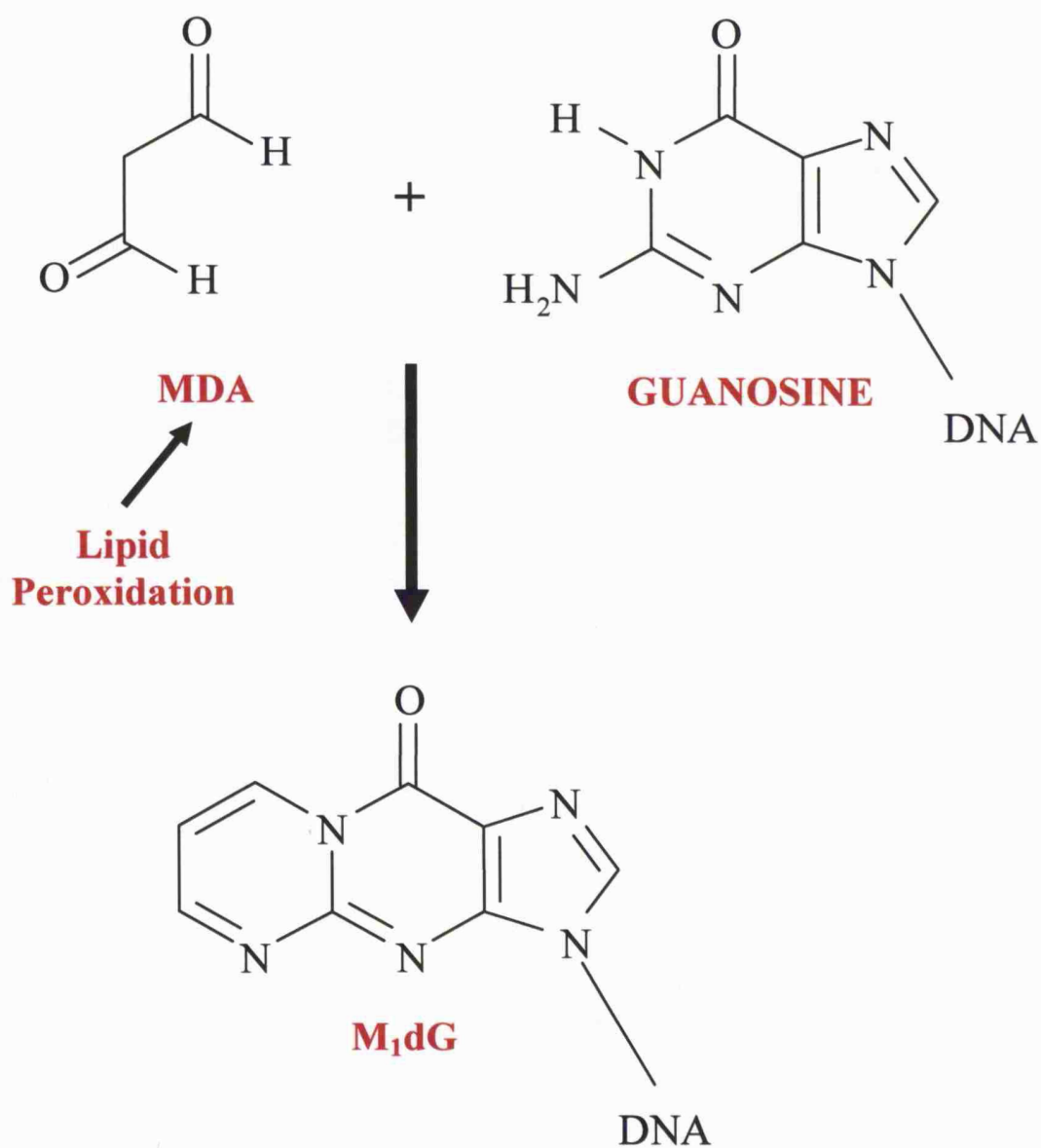


Figure 1.3.2 Chemical structure of 8-oxo-dG. The production of the highly reactive oxygen species, hydroxyl anion (OH^-), subsequently reacts with 2'-deoxyguanosine producing the oxidative DNA adduct, 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxo-dG).

Oxidative DNA adduct: M₁dG

Initial interest in M₁dG adducts arose from the knowledge that MDA is generated during lipid peroxidation and the potential link between lipid peroxidation and carcinogenesis. This hypothesis has been supported by a number of studies. It has been shown that M₁dG levels were 3-fold higher in histologically normal breast tissue of women with breast cancer than in normal breast tissue of women without breast cancer (Wang *et al*, 1996). Colorectal adenomas are considered pre-malignant lesions of colorectal cancer. Leuratti *et al.* (2002) found higher levels of M₁dG in normal colonic mucosal biopsies in patients found to have adenomas in their colon. There is increasing evidence that M₁dG may serve as a biomarker for chemoprevention studies of colorectal cancer. In the Apc^{min+} mouse, a model of human familial adenomatous polyposis (FAP), M₁dG levels were significantly reduced in intestinal adenomas of mice treated with the strongly anti-oxidant chemopreventive agent, curcumin, compared with the controls (Tunstall *et al*, 2006). In a clinical pilot study of patients undergoing surgery for colorectal cancer, M₁dG levels were found to be 2.5-fold higher in tumour tissue than normal mucosa. Furthermore, the M₁dG levels were found to have reduced significantly in post-surgery tumour tissue compared to pre-surgery tumour tissue in patients treated with curcumin for 7 days prior to surgery (Garcea *et al*, 2005).

Figure 1.3.3 Chemical structure of M₁dG formation. Lipid peroxidation results in the production of malondialdehyde (MDA), this then reacts with guanosine, a base moiety of DNA. This reaction leads to the production of the oxidative DNA adduct, M₁dG (cyclic pyrimidopurinone N-1, N² malondialdehyde-2'-deoxyguanosine).



1.3.1 Lipid metabolism and oxidative stress

Diets rich in omega-3 polyunsaturated fatty acids (eicosapentaenoic acid, docosahexaenoic acid) found in fish oils may reduce the risk of prostate cancer (Norrish *et al*, 1999). Omega-6 polyunsaturated fatty acids such as arachidonic acid and its precursor, linolenic acid, are major ingredients of vegetable oils. They are consumed in high quantities in the typical Western diet and may be relevant to the pathogenesis of prostate cancer (Godley *et al*, 1996). However, a comprehensive review by Astorg *et al*. (2004) found little evidence to support the association of both omega-3 and omega-6 polyunsaturated fatty acids with prostate cancer. Oxidative lipid peroxidation accompanying the biosynthetic metabolism of arachidonic acid from membrane phospholipids to prostaglandins is a likely mechanism in prostate carcinogenesis (Mukai and Goldstein, 1976). Cyclooxygenase is a rate-limiting enzyme in prostaglandin biosynthesis, a two-step enzymatic process in which ROS are generated (Nikolic and van Breemen, 2001). Firstly, arachidonic acid is converted to prostaglandin G2 by the oxygenase activity of cyclooxygenase, prostaglandin G2 then undergoes peroxidation to prostaglandin H2. Unlike cyclo-oxygenase-1 (COX-1), COX-2 is normally undetectable in most tissues, but it is induced by a variety of stimuli including mitogens, growth factors and cytokines (Wadleigh *et al*, 2000). Recent studies have shown that over expression of COX-2 is sufficient to induce breast tumours in transgenic mice. Subsequently, inhibition of the COX-2 pathway resulted in reduction in tumour incidence and progression (Liu *et al*, 2001). In prostate cancer, COX-2 expression assessed by immunohistochemistry (Kirschenbaum *et al*, 2000; Madaan *et al*, 2000; Yoshimura *et al*, 2000), Western

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blotting (Zha *et al*, 2001) and reverse transcription polymerase chain reaction (Gupta *et al*, 2000) is significantly higher in prostate cancer tissue compared with benign prostatic tissue. The intensity of immunoreactivity of COX-2 correlates with tumour grade (Lee *et al*, 2001). Of particular interest, increased immunostaining for COX-2 has also been seen in the majority of HGPIN (Uotila *et al*, 2001), and PIA lesions (Zha *et al*, 2001). In contrast, other investigators have failed to detect COX-2 in human prostate cancer cell lines commonly used in mechanistic studies, such as PC3, LNCaP and DU145 (Subbarayan *et al*, 2001). Despite the limitations of *in vitro* model systems in studying the link between COX-2 and oxidative DNA damage, this would make the use of COX-2 inhibitors an attractive option in the chemoprevention of prostate cancer *in vivo* (Sharma *et al*, 2001).

Epidemiological observations support the importance of NSAIDs in the chemoprevention of colon cancer (DuBois *et al*, 1996). However, the epidemiological evidence for a protective effect of NSAIDs against prostate cancer is equivocal (Nelson and Harris, 2000; Norrish *et al*, 1998). *In vitro* studies using LNCaP and PC3 cell lines show that NSAIDs, such as sulindac derivatives and etodolac, decrease cell growth and induce apoptosis (Kamijo *et al*, 2001; Lim *et al*, 1999). In contrast, apoptosis was not demonstrated in normal prostatic epithelial (PrEC) and stromal (PrSC) cell lines (Kamijo *et al*, 2001; Lim *et al*, 1999). *In vivo* studies have shown that selective COX-2 inhibitors can induce apoptosis in PC3 cells grown in nude mice (Liu *et al*, 2000). However, it is important to recognise that some of the effects of NSAIDs and selective COX-2 inhibitors are independent of COX-2 activity. Indeed, derivatives of celecoxib

which lack COX-2 inhibitory activity can induce apoptosis in PC3 cells (Song *et al*, 2002). Multiple events peripheral to COX-2 that may be relevant to induction of apoptosis are under scrutiny, including the dephosphorylation of Akt and the MAP kinase, Erk2 (Zhu *et al*, 2002).

1.3.2 Anti-oxidant enzymes and oxidative stress

As briefly mentioned above, GST- π is a major anti-oxidant isoenzyme found in cells. Glutathione conjugates ROS catalysed by GST- π . The expression of the *GSTP1* gene has been found to be increased in PIA lesions (De Marzo *et al*, 1999), suggesting a mechanism to prevent oxidative damage. However, it is seen to be inactivated in approximately 70% of HGPIN lesions, and greater than 90% of prostate cancers analysed (Brooks *et al*, 1998; Lee *et al*, 1997). Thus, the protective mechanisms against oxidative damage in prostate cells are reduced as lesions progress from pre-malignant to malignant. Therefore, it is conceivable that intervention with anti-oxidants may combat prostate cancer both in the early and advanced stages.

1.3.3 Androgens and oxidative stress

Mechanistically, it was in the late 1990's that a link between ROS and androgens was made. Experimental work carried out on the androgen-sensitive human prostate cancer cell, LNCaP, found that stimulation with physiological and supra-physiological levels of DHT resulted in increased levels of ROS (O'Ripple *et al*, 1997). Furthermore, the increase in ROS was proportional to the dose of DHT used. Further work carried out by Sun *et al*. (2001), has supported this initial work. This group showed that androgens increase ROS, however, in the presence of the competitive androgen receptor antagonist, flutamide, ROS generation was inhibited. This study also showed that by blocking the action of PSA using an anti-PSA antibody, androgen-induced ROS was inhibited. As would expected, androgen stimulation of the human prostate cancer cell lines, DU145 and PC3, which are both androgen-insensitive, does not appear to influence levels of ROS (O'Ripple *et al*, 1997; Sun *et al*, 2001).

The largest chemoprevention trial completed to date is the PCPT. Currently, the REDUCE trial is underway. Both of these chemoprevention trials are based on the notion that androgen deprivation leads to a reduction in the incidence of prostate cancer. Therefore, exploring the role of androgens and oxidative stress may lead to further insight into prostate carcinogenesis.

1.4 Pre-clinical models of prostate carcinogenesis

Unfortunately, little understanding exists regarding prostate cancer initiation and progression to the malignant phenotype. The uncertain time course and heterogeneity of the disease hinder elucidation of prostate carcinogenesis. This makes the procurement of clinical specimens for the study from appropriate time points during prostate tumour progression largely impossible (Schwab *et al*, 2000). Fundamental to improving our understanding of the molecular and cellular events of prostate carcinogenesis is the need to develop relevant pre-clinical models.

1.4.1 Human prostate cell lines

Historically, *in vitro* cultures of human prostate cells have been limited in availability and scope compared with those from many other organs (Peehl, 2005). The three commonly used human prostate cells lines are PC-3, DU145 and LNCaP. Although other prostate cancer cell lines are available, recent studies revealed that many of these are in fact, derivatives of the three aforementioned cell lines (Van Bokhoven *et al*, 2003). Derivation of PC-3, DU145 and LNCaP cell lines resulted from the immortalisation of metastatic human prostate cancer cells (Horoszewicz *et al*, 1980; Kaighn *et al*, 1979; Stone *et al*, 1978) summarised in Table 1.5.1.1. PC3 and DU145 cell growth is independent of androgens whilst LNCaP cells are androgen-sensitive. Unlike PC-3 and DU145, LNCaP cells also produce PSA. Thus, the LNCaP cell line provides a pre-clinical model for assessing the molecular and cellular events that are androgen-dependent. Using these cell lines to understand the early events of prostate carcinogenesis has clear limitations. Cell lines more appropriate for the study of early prostate cancer

would be those derived from spontaneously immortalised prostate cancer cells. Unfortunately, explanted prostate cells survive only short-term in culture and rarely immortalise spontaneously. The use of viral transforming proteins to immortalise normal and primary tumour-derived human prostatic epithelium has allowed the continual propagation of these cells *in vitro* (Bright *et al*, 1997).

PROSTATE CANCER CELL LINE	ORIGIN	ANDROGEN SENSITIVITY
DU145	Caucasian 69 year old male Brain metastasis	No
PC3	Caucasian 62 year old male Bone metastasis	No
LNCaP	Caucasian 50 year old male Lymph Node metastasis	Yes

Table 1.5.1.1 Origins and characteristics of human prostate cancer cells.

1.4.2 Animal models

The use of animal models of prostate cancer is critically important for defining the molecular basis of the disease and also to accelerate the development of new chemopreventive approaches and therapies for prostate cancer (Lamb and Zhang, 2005). Important physiological processes that are lacking *in vitro* include three-dimensional structure, angiogenesis, stromal interaction influencing both tumour development, growth and metastatic spread (Van Weerden and Romijn, 2000).

Mouse models

Xenograft mice

In immunodeficient mice, tumour growth occurs after implantation of human cancer cell lines with no graft-versus-host response. The severe immunodeficient mouse (SCID) model lack T- and B-lymphocyte function (Bosma *et al*, 1983). This allows human prostate cancer cells to be implanted subcutaneously (xenograft) or intra-prostatically (orthotopic). Athymic nude mice have a congenital absence of the thymus gland and thus, are only deficient in matured T-cell immunity (Fidler, 1986). Obvious drawbacks of xenograft models are that the tumour and target organ are not from the same species and the study of initiating events in prostate carcinogenesis is precluded. Nevertheless, these models appear to have considerable utility for the testing of chemopreventive agents as well as diverse therapeutic agents (Stearns *et al*, 1998).

Transgenic mice

The use of viral oncogenes (e.g. SV40) is commonly used to induce prostate cancer in transgenic mouse models. The Transgenic adenocarcinoma mouse prostate (TRAMP) mouse is one such model (Gingrich *et al*, 1996; Greenberg *et al*, 1995). Various stages of progressive prostate disease were observed in the TRAMP model; focal adenocarcinoma developing with 100% frequency in mice between 10 and 20 weeks of age (Greenberg *et al*, 1995). The advanced stages of human prostate cancer were also mimicked to some extent with metastatic deposition in lymph nodes and bone (Gingrich *et al*, 1996). This model has already been used for chemoprevention studies of green tea and genistein (Adhami *et al*, 2003; Mentor-Marcel *et al*, 2005).

Rodent models

The Lobund-Wistar, Dunning and Noble rat models (Dunning, 1963; Pollard, 1998) have been used extensively to study hormonal carcinogenesis. However, the limitations of these models include; long tumour latencies, stochastic variability and lack of spontaneous metastases and have largely been supplanted by the mouse models mentioned above.

Canine models

Apart from man, the dog is the only species that develops prostate cancer spontaneously (Waters *et al*, 1996; Waters and Bostwick, 1997). The similarities with human prostate cancer include; heterogeneity, age at diagnosis, association with HGPIN and propensity for bone metastasis. However, unlike humans, the tumours that form are androgen-independent and lack a functional AR. Therefore, the dog represents an important model for studies of advanced prostate cancer. Major limitations of the canine model include long latency period related to the lifespan, size and maintenance costs (Navone *et al*, 1998).

Most animals and non human primates do not spontaneously develop prostate cancer. Accordingly, the relevance of any animal model to human disease must still be considered with reservations.

1.5 Aims

The principal aim of this project was to determine the role of oxidative DNA adducts as potential biomarkers to be used in pilot/pre-clinical trials of chemoprevention of prostate cancer.

Reactive oxygen species are known to be factors of importance in the development and progression of prostate cancer (Section 1.3). They may cause peroxidation of membrane lipids and oxidative DNA damage, resulting in the formation of oxidative DNA adducts, which have been associated with carcinogenesis. It has been shown that androgens increase ROS in human prostate cancer cells cultured *in vitro*. However, such species are unstable, short lived and technically difficult to measure without artefact. We tested a number of hypotheses based on the concept that androgens increase ROS, which may result in oxidative DNA damage, leading to the formation of oxidative DNA adducts. These adducts are known to be more stable and technically easier to measure than ROS. The oxidative DNA adducts measured were M₁dG and 8-oxo-dG. We tested hypotheses in two pre-clinical model systems: LNCaP, DU145 and PC3 cells grown *in vitro* and the LNCaP cells grown as a xenograft in athymic nude mice.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 General chemicals and kits

All chemicals and reagents were purchased from Sigma-Aldrich Company Ltd (Poole, UK). Unless otherwise stated, all solvents were purchased from Fischer Scientific (Loughborough, UK).

2.1.2 Cell Culture

Human prostate cancer cells, DU145, PC3 and LNCaP cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK).

Cell culture media, RPMI 1640 (25 mM Hepes) was purchased from Sigma (Poole, UK) and MEM Eagles from Gibco (Paisley, UK). Trypsin, glutamax and foetal calf serum were also purchased from Gibco (Paisley, UK). Glucose, sodium pyruvate, dihydrotestosterone and flutamide were purchased from Sigma (Poole, UK). Bleomycin was purchased from Calbiochem (Germany).

Cell culture flasks (with filter caps) were purchased from Nunc (Roskilde, Denmark).

2.1.3 Growth Curves

24 well-plates were purchased from Nunc (Roskilde, Denmark). Isoton II buffer solution was purchased from Bomi Ltd (High Wycombe, UK). The cell counts were performed by using the Z2 Beckmann Coulton™ counter.

2.1.4 Determination of M₁dG levels

Genomic DNA extraction was performed using reagents and columns obtained from Qiagen (Hilden, Germany). Chemiluminescence substrates were purchased from Supersignal Ultra (Rockford, USA). Propidium Iodide was purchased from Sigma (Poole, UK). Dipotassium hydrogen-orthophosphate and ammonium acetate were purchased from Fisher chemicals (Cambridge, UK). Murine M₁dG monoclonal antibody D10A1 (primary antibody) was kindly donated by Professor Larry Marnett (Department of Biochemistry, Vanderbilt University, USA). M₁dG standards and sodium MDA, the sodium salt of monomeric MDA, was synthesised and characterised by Dr R Singh (Department of Biochemistry, University of Leicester). Polyclonal goat anti-mouse antibody (secondary antibody) was purchased from Dako Pharmaceuticals (Cambridge, UK). Immunoblot apparatus, nitrocellulose and filter paper were purchased from Schleicher and Schuell Keene (Dassel, Germany).

2.1.5 Reagents and enzymes used in the extraction of DNA for the determination of M₁dG levels

Cell Lysis Buffer (C1)

- 1 mM sucrose, 3 mM magnesium chloride, 10% TRIS, 100% triton X-100, pH 7.0 (store at room temperature).

General Lysis Buffer (G2)

- 800 mM guanidine hydrochloride, 30 mM TRIS-Chloride, 30 mM ethylenediamine tetraacetic acid, 5% Tween-20, 0.5% Triton X-100, pH 8.0.

Equilibration Buffer (QBT)

- 750 mM sodium chloride, 50 mM 3-(n-morpholino) propanesulfonic acid (MOPS), 15% isopropanol, 0.15% triton X-100, pH 7.0.

Wash Buffer (QC)

- 1.0 M sodium chloride, 50 mM MOPS, 15% isopropanol, pH 7.0.

Elution Buffer (QF)

- 1.25% sodium chloride, 50 mM TRIS-Chloride, 15% isopropanol, pH 8.5.

2.1.6 Determination of 8-oxo-dG levels

TRIS base, sucrose, magnesium chloride, desferoxamine mesylate, sodium EDTA, triton X-100, RNAase, DNAase and snake venom phosphodiesterase were purchased from Sigma (Poole, UK). Shrimp alkaline phosphatase was purchased from Amersham Biotech Ltd (Chalfont, UK). Protease was purchased from Qiagen (Hilden, Germany). The centrifugal filter device (10,000 MW, YM-30) was purchased from Millipore (Bedford, USA). The immunoaffinity column was prepared and obtained from Dr R Singh.

2.1.7 Reagents and enzymes used in the extraction of DNA for the determination of 8-oxo-dG

Buffer A

10 mM Tris, 320 mM Sucrose, 5 mM magnesium chloride, 0.1 mM desferoxamine mesylate, 1% triton X-100, pH 7.5 and stored at -20°C.

Buffer B

10 mM Tris, 5 mM sodium ethylenediamine tetraacetic acid, 0.15 mM desferoxamine mesylate, pH 8.0 and stored at -20°C.

Sodium Iodide

40 mM Tris, 20 mM sodium ethylenediamine tetraacetic acid, 3 mM desferoxamine mesylate, 7.6 M sodium iodide puriss, pH 8.0 and stored at 4°C.

Protease

Concentration 20 mg/ml and stored at 4°C.

RNAase IIIA

10 mM Tris, concentration 100 mg/ml, pH 8.0 and stored at -20°C.

RNAase T1

10 mM Tris, concentration 1U/ μ l, pH 8.0 and stored at -20°C.

Digestion buffer (DB).

40mM TRIS-HCl, M MgCl₂, pH 8.5

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Shrimp Alkaline Phosphatase (SAP).

Concentration of 1U/ μ l.

Deoxyribonuclease I (DNase I).

DNase (Bovine Pancreas) dissolve in HPLC water at a concentration of 4U/ μ l.

Snake Venom Phosphodiesterase (SVP).

SVP obtained from *Crotalus atrox*, dissolved in HPLC water at a concentration of 0.00323U/ μ l.

70% Ethanol.

10% Sodium Dodecyl Sulphate (SDS).

40% and 100% Isopropanol (analytical grade).

0.1 mM Desferoxamine mesylate.

2.1.8 Animals and Diets

Outbred male athymic nude mice 30-40g (MF1) were purchased from Harlan (Bicester, UK). High protein diet, RM3 pellets were purchased from Special Dietary Services (Witham, UK). Matrigel was purchased from BD Biosciences (Bedford, USA). Halothane gas was purchased from Concord Pharmaceuticals (Dunmow, UK).

2.2 *In Vitro* Methods

2.2.1 Establishment of cell cultures from frozen cells

Frozen cells stored in liquid nitrogen (-80°C) were removed and gently thawed in a water bath at 37°C. The cells were re-suspended in 10 mls of fresh cell culture medium. The cell suspension was then centrifuged at 350 x *g* for 5 mins at 4°C. The supernatant was discarded and cells were re-suspended in 10 mls fresh cell culture medium and transferred into Nunc T25cm² cell culture flasks. Cell cultures were maintained in a humidified atmosphere of 5% carbon dioxide (CO₂) at 37°C. Cells were examined daily under light microscopy. Once cells reached confluence of 80-90%, cells were passaged as described below.

2.2.2 Routine passage of cells

The T25cm² cell culture flasks were removed from the incubator and the culture medium was discarded. Cells were washed with Phosphate Buffered Saline (PBS) to remove non-adherent dead cells and traces of culture medium. An aliquot of 5 mls trypsin (1%) was added to the flasks and flasks transferred back into the incubator for 5 mins. Flasks were gently tapped to detach the remaining adherent cells before 5 mls of fresh medium was added to neutralise the effects of trypsin. The cell suspension was then centrifuged at 350 x *g* for 5 mins at 4°C. The supernatant was discarded and the residual pellet was re-suspended in 5 mls of fresh medium. An aliquot of 50 µls of this suspension was then mixed with 50 µls of trypan blue solution and the cells counted using a microscopic counting chamber, the haemocytometer. Cells were seeded in T175cm² flasks at a density

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of 1×10^6 in 50 mls fresh medium. Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

2.2.3 Determination of growth characteristics of LNCaP cells

LNCaP cells were grown as discussed above. Following harvesting and cell counts, 1×10^4 LNCaP cells in 1 ml of cell culture medium were seeded into each 24 well plate. The cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Cells were allowed to settle in the wells for 24 hours. Subsequently, a further 1 ml of cell culture medium was gently added to each well. The cell culture medium was changed in each well at 96 and 144 hrs. Daily treatments with either DHT, flutamide or the combination of the two were carried out. Unless otherwise stated, cell counts were performed at 72 and 144 hrs. Aliquots of 0.5 mls of trypsin (1%) were added to each well and incubated for 5 mins at 37°C. After this time, 0.5 mls of fresh cell culture medium was added to neutralise the trypsin. The cell suspension of 200 µls was added to 9.8 mls of isoton solution. Cell counts were performed using Coulter Z2 particle counter set between the range of Tmax 11.9 µM and Tmin 10.0 µM.

2.2.4 Cell culture with DHT and Flutamide

Human prostate cancer cell lines used were DU145, PC3 and LNCaP; they were grown in cell culture containing 10% foetal calf serum (FCS), unless otherwise stated. The cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. DU145 cells were grown in MEM Eagles media containing 0.2% non-essential amino acids (NEAA) and 1% sodium pyruvate. PC3 cells were grown in RPMI 1640 media containing 1% glutamax. LNCaP cells were grown in RPMI

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1640 media containing 1% glutamax and 1% sodium pyruvate. Unless otherwise stated, cells were grown in T175cm² flasks. Ethanol (100%) was used both to suspend DHT and flutamide in solution and act as vehicle-control. In combination studies, flutamide was added to the cells 30 minutes before DHT. During the 7 day experiments, culture media was changed twice a week. The cells were harvested with trypsin (1%) and cell counts were performed using a haemocytometer. All experiments were carried out in triplicate, unless otherwise stated.

2.2.5 Cell culture with Bleomycin

LNCaP cells were grown in T175cm² flasks as described in section 2.2.2. The media was changed to serum-free on day 5. Bleomycin was dissolved in water (avoidance of light). Cells were then treated with bleomycin at varying concentrations and duration. The cells were harvested with trypsin (1%) and cell counts were performed using a haemocytometer. Subsequently, DNA was extracted from the cells and M₁dG levels were determined as described in section 2.3.

2.3 DNA extraction from cells for the determination of M₁dG levels

DNA extraction from human prostate cancer cells was performed for M₁dG analysis using the Qiagen method (Figure 2.3.1).

- Cells were harvested from T175 cm² with 10 mls of trypsin (1%)
- Cell suspensions were centrifuged in 50 ml falcon tubes containing 40 mls of cell media, at 350 x g for 5 mins at 4°C. The supernatant was discarded and the cell pellet was re-suspended initially in 2 mls of cell media followed by further dilution in 5-8 mls of cell media.
- Cell counts were performed using trypan blue and the haemocytometer.
- Ice cold PBS was added to the cell suspension to make up to a final volume of 50 mls. The suspension was centrifuged at 1500 x g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was again re-suspended in 2 mls of PBS.
- 2 mls of ice cold C1 lysis buffer and 6 mls of ice cold water (HPLC grade) were added. The sample was inverted several times and then incubated for 10 minutes on ice (4°C).
- Suspension was centrifuged at 1300 x g for 15 minutes at 4°C.
- Supernatant was discarded and the pellet re-suspended in 1 ml of C1 lysis buffer and 3 mls water, following gentle vortex the sample was centrifuged at 1300 x g for 15 minutes at 4°C.
- Supernatant discarded and the nuclear pellet completely re-suspended in 5 mls G2 buffer by gentle vortex.

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- 95 μ ls of Proteinase K and 162 μ ls of RNAase A were added, followed by incubation for 2 hours at 37°C in water bath.
- 100/G Qiagen genomic-tip was equilibrated with 4 mls of QBT buffer.
- The sample was added to the genomic-tip followed by two washes with 7.5 mls of QC buffer.
- Elution of DNA from the genomic-tips with 5mls of QF elution buffer (50°C) into 15 ml falcon tubes containing 3.5 mls of isopropanol (100%).
- DNA was precipitated by gently inverting the tubes 10-20 times, followed by centrifugation at 4000 x g for 20 minutes at 4°C.
- Supernatant was discarded and the pellet washed with 0.5-1.5 mls of ethanol (70%), then transferred into a 2 ml eppendorf tube.
- Sample centrifuged at 5000 x g for 10 minutes at 4°C.
- Supernatant was discarded and the pellet was allowed to dry for a few minutes at room temperature.
- DNA pellet was dissolved in 200 μ ls of water and then stored at -80°C.

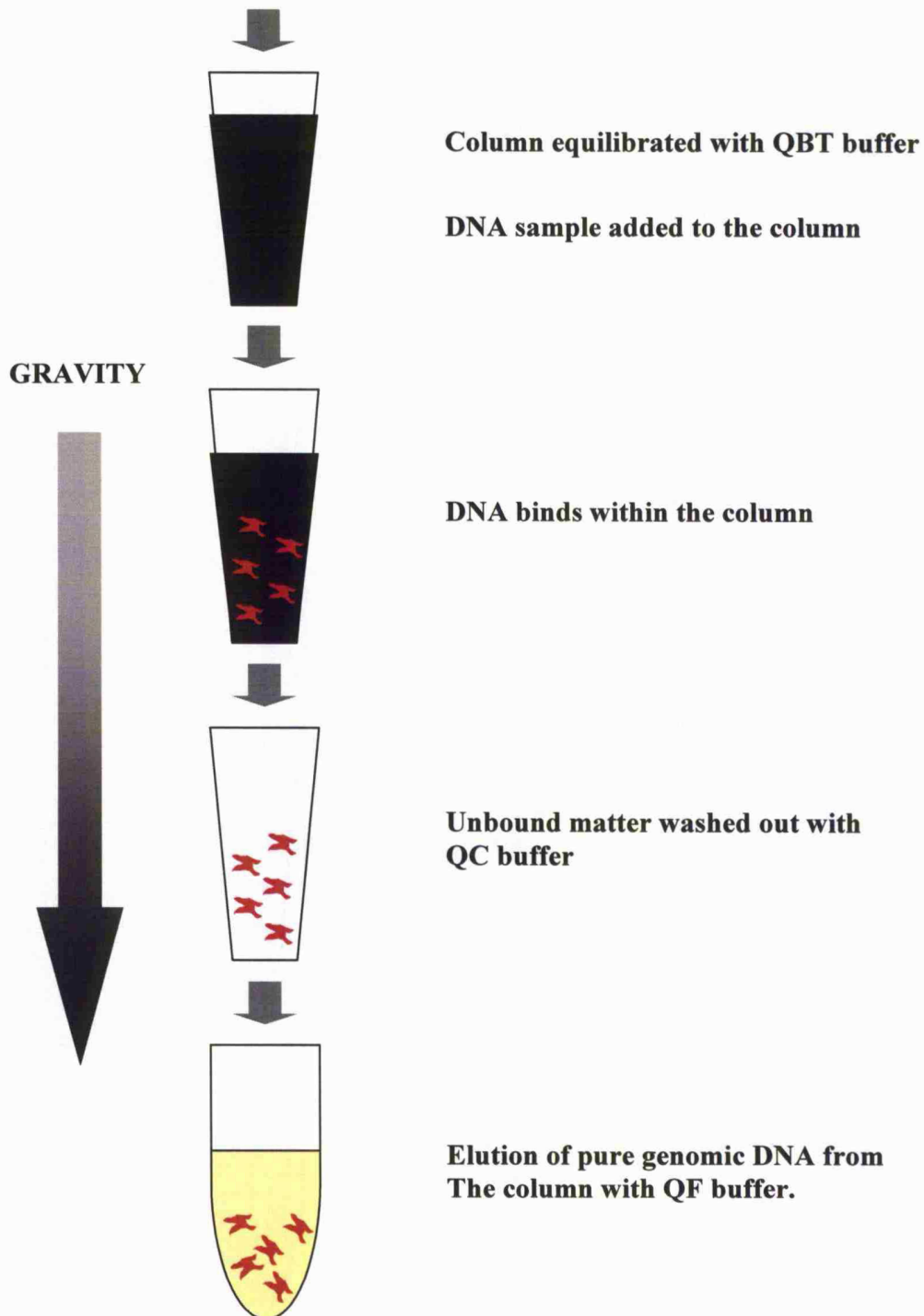


FIGURE 2.3.1 Qiagen method used for the extraction of DNA. The figure illustrates the steps involved in the extraction and isolation of pure genomic DNA from human prostate cancer cells (DU145, PC3 and LNCaP) and murine xenograft tissues (blood, prostate and LNCaP tumours). Subsequently, the DNA is used for the determination of M₁dG levels.

2.3.1 DNA extraction from blood and tissues for the determination of M₁dG levels.

DNA extraction was performed using the Qiagen Genomic Column and Buffer kit. Different quantities of buffer solutions, enzymes and column sizes were used depending on the volume of tissue used. For samples less than 20 mg in weight, the 20/G Qiagen columns were used and the volume of buffers and enzymes are shown in bold. For samples weighing 20-100 mg the 100/G column were used and the volumes of buffers and enzymes are shown in italics.

- **4µl**, *19 µl*, of RNAse A solution was added to **2 ml**, *5 ml* of G2 buffer and thoroughly homogenised with the tissues.

The homogenate was transferred to **10 ml**, *50 ml*, screw-cap tubes and **0.1 ml**, *0.5ml*, of the Qiagen protease was added. The homogenate was thoroughly vortexed and incubated at 50 °C for 1 hour.

The homogenate was then centrifuged at 5000 x g for 10 minutes at 4°C to remove excess residue and the supernatant transferred into the Qiagen tips (see below).

- The 20/G, 100/G Genomic tips, were equilibrated with **1 ml**, *4 ml* of QBT buffers.
- The homogenates were vigorously vortexed for 10 seconds and then transferred into the equilibrated tips.
- The tips were then washed with **3 x 1ml**, *2 x 7.5 ml* of QC buffers.

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- The DNA was then eluted from the column with 2 x 1 ml, 1 x 5 ml of QF buffers. The DNA was collected and precipitated in 1.4 ml, 3.5 ml of isopropanol (100%) at room temperature.
- The samples were then immediately centrifuged at > 5000 x g for at least 15 minutes at 4°C, the supernatants carefully removed, leaving the DNA pellets at the bottom of the tubes.
- The centrifuged DNA pellets were washed with 1 ml of 70% ethanol solution at room temperature and transferred into eppendorf tubes. The pellets were briefly vortexed and the centrifuged at > 5000 x g for 10 minutes, the 70% ethanol solution was carefully removed, leaving the pellets at the bottom of the eppendorfs. The pellet was allowed to dry at room temperature for a few minutes and then re-suspended in 100 – 200 µl of HPLC grade water, gently vortexed, then stored at -80°C.

2.3.2 Ultra-violet spectrophotometry

The DNA sample was taken and diluted by a factor of 200 in HPLC grade water. The sample was then placed in a quartz cuvette and analysed, the quantity and purity of genomic DNA sample was determined by uv spectrophotometry using the 260/280 nm ratio (range 1.6-1.9). This was used to calculate the volume of DNA sample required to give 3.5 µg of DNA for M₁dG determination.

$$\begin{array}{cccc} \text{[DNA sample]} & = & \text{absorbance} & \times & \text{dilution factor} & \times & C \\ (\mu\text{g}/\mu\text{l}) & & (260 \text{ nM}) & & (200) & & (0.05) \end{array}$$

2.3.3 Quantification of the oxidative DNA adduct, M₁dG by immunoslot blot

Levels of M₁dG were determined by the immunoslot blot method previously described (Lauratti *et al.*, 1998). Figure 2.3.3.1 summarises the method. The series of samples and standards were performed in triplicate; the latter were used to determine the calibration curve (Figure 2.3.3.3 and 2.3.3.4). Equal loading was determined by propidium iodide staining following adduct measurement (Singh *et al.*, 2001). The intensity of adduct staining was measured using a Genegnome™ fluorescence imager. The actual M₁dG levels were calculated and expressed as adducts per 10⁷ nucleotides from the calibration curve.

- Calf thymus DNA was diluted with MDA-treated calf thymus DNA to obtain a series of standards with 5 µg DNA in a volume of 50µls HPLC-grade water. The diluted standards are listed below.

Sample	control calf thymus DNA (µls)	MDA treated calf thymus DNA ((µls)	M ₁ dG (fmol)
1	50	0	0.2
2	48	2	0.4
3	45	5	1.0
4	40	10	2.0
5	35	15	3.0
6	25	25	5.0
7	20	30	6.0
8	10	40	8.0
9	0	50	10.0

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- Following gentle vortexing of the above standards, 35 μs (3.5 μg) of each standard was transferred into a 1 ml eppendorf containing 65 μs of KP buffer, followed by the addition of 150 μs of PBS. Samples were then gently vortexed.
- Each specimen DNA sample was transferred into 1 ml eppendorfs; volume equivalent to 3.5 μg of DNA, KP buffer was added to make a final volume of 100 μs . Finally, 150 μs of PBS was added and the samples gently vortexed.
- The standard and specimen DNA were then centrifuged at 14,000 rpm for 1 minute.
- Followed by sonication of DNA for 20 minutes, thus allowing the DNA strands to be broken down into 100 base pairs long.
- Samples were then heated for 5 minutes at 100°C, thus allowing double stranded DNA to form single strands.
- Eppendorfs placed on ice for at least 10 minutes.
- 250 μs of 2M ammonium acetate was added to each eppendorf, gently vortexed and centrifuged at 14,000 rpm for two minutes.
- Immunoslot blot apparatus was set up and the suction aspirator turned on (Figure 2.3.3.2)
- A volume of 142.86 μs of standards and specimen was added to the wells in triplicate and the wells were allowed to run dry.
- To each well 200 μs of 1M ammonium acetate was added and again the wells were allowed to run dry, aspirator remained “on” for a further 15 minutes.

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- The nitrocellulose filter paper was removed and baked in a pre-heated vacuum oven at 80°C for 1.5 hours
- The filter paper was then washed in 100 mls of PBS-T containing 5% milk powder for 1 hour at room temperature.
- The filter paper was then washed twice in 50 mls PBS-T for 5 minutes.
- The filter paper was then incubated in 40 mls of PBS-T containing 0.5% milk powder and 5 µls of primary antibody (M₁dG) and gently rocked for 2 hours at room temperature. Followed by gentle rocking overnight in the cold room (4°C).
- The filter paper was then washed with 50 mls of PBS-T for 1 minute, followed by two further 5 minute washes.
- The filter paper was then incubated in 32 mls of PBS-T containing 0.5% milk powder and 8µls of secondary antibody (goat anti-mouse) and rocked for 2 hours at room temperature.
- The filter paper was then washed in 50 mls PBS-T for 15 minutes followed by two further 5 minute washes.
- The supersignal ultra chemiluminescence reagent working solution was prepared by mixing equal volumes (4 mls) of the ultra enhancer solution with the ultra stable peroxide solution.
- The filter was bathed in 8 mls of Chemiluminescence reagent for 5 minutes at room temperature.
- The filter was gently blotted onto a paper towel prior to ECL analysis in the Genegenome™ Bioimaging system.

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- Following detection of the M₁dG adducts, the filter was washed overnight in 50 mls PBS solution.
- The filter was then bathed in 50 mls of PBS containing 50 µls of Propidium iodide and gently rocked for 3 hours at room temperature. (Protected from sunlight by placing container in black box).
- The filter paper was then washed in 50 mls of PBS initially for 1 hour, followed by a further wash for 30 minutes.
- The image was then captured on the Genegnome™ Bioimaging system.
- The M₁dG adduct levels were corrected on the base of PI staining of each band and were expressed as adducts/10⁷ nucleotides.

Double stranded DNA

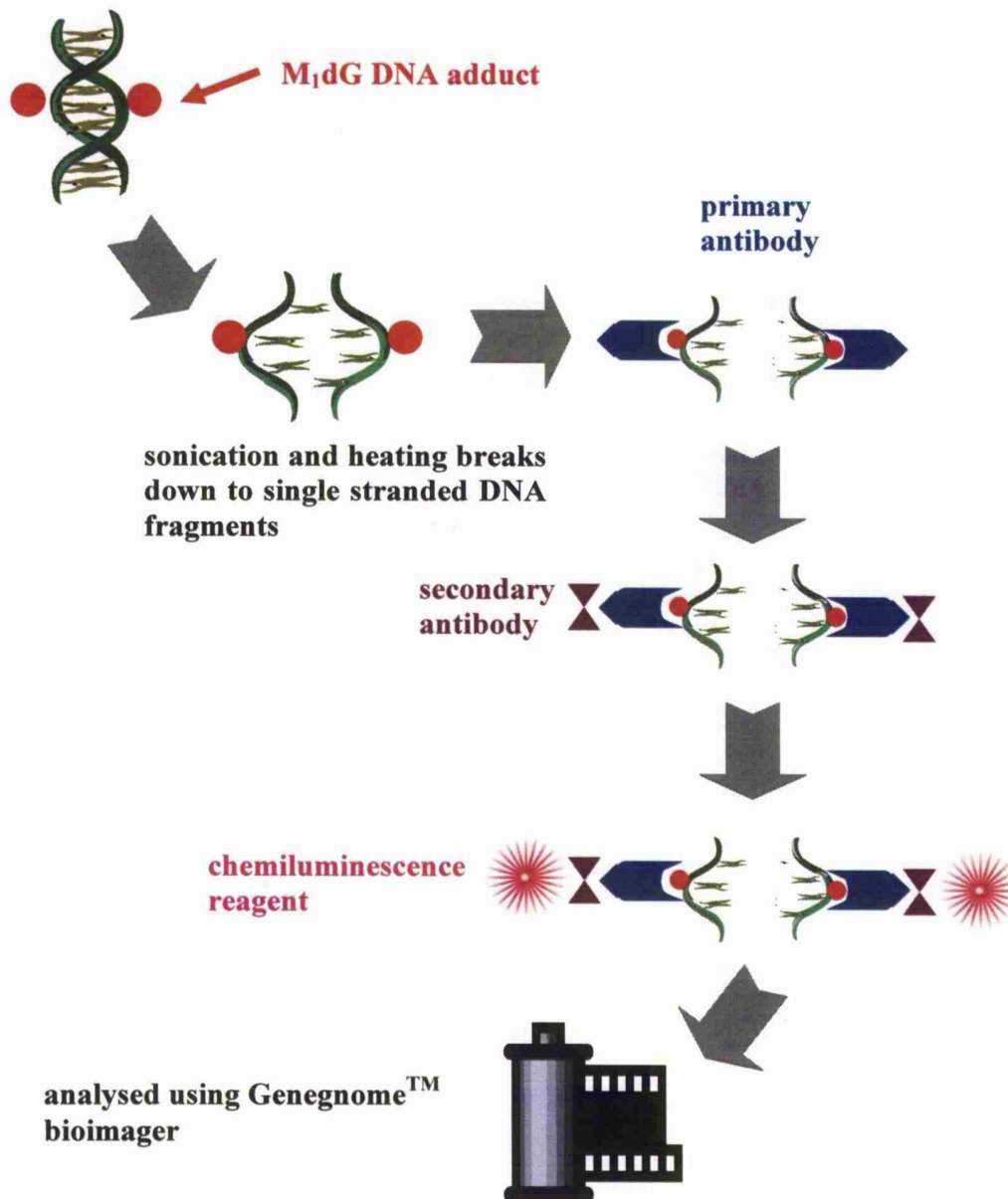


FIGURE 2.3.3.1 Immunoslot blot method used to determine M₁dG levels. The schematic representation shows the steps involved in the determination of M₁dG levels in pure genomic DNA. The pure genomic DNA used had previously been extracted from human prostate cancer cells (DU145, PC3 and LNCaP), murine tissues (blood, prostate) and xenograft LNCaP tumours using the Qiagen method.

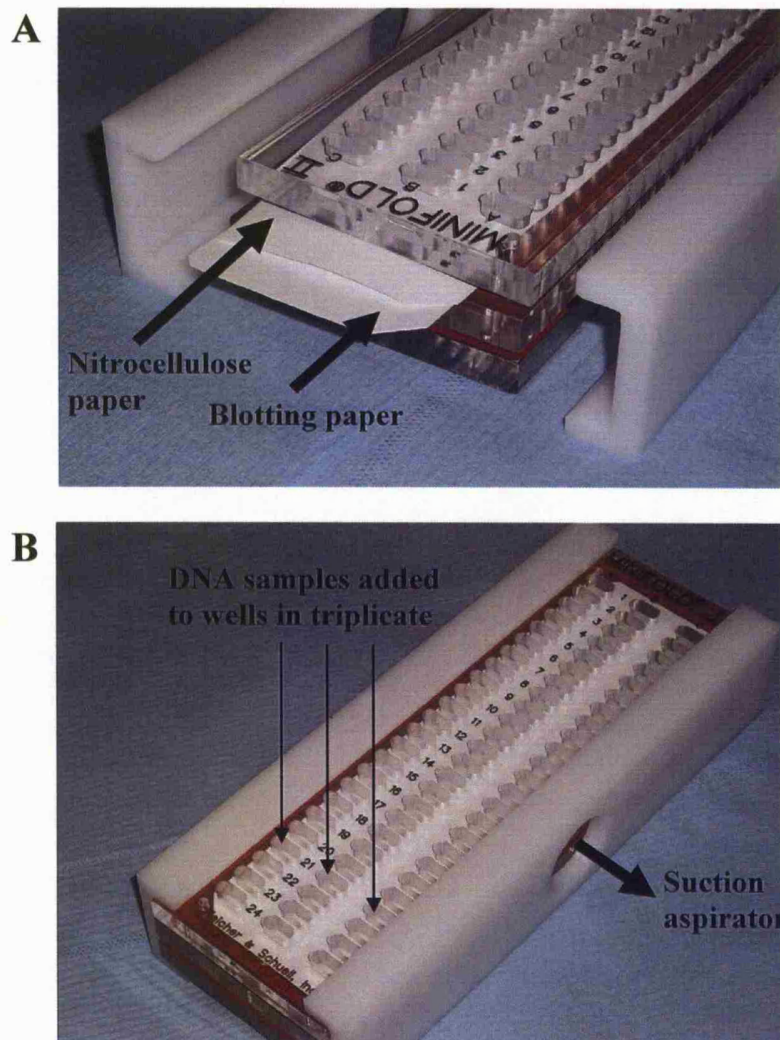


Figure 2.3.3.2 Immunoblot apparatus. The nitrocellulose paper was bathed in HPLC water and then laid upon the blotting paper that had been pre-bathed in [1M] ammonium acetate (A). Immunoblot apparatus was assembled and DNA samples were added to each well in triplicate during suction aspiration of the wells (B). Control DNA samples (calf thymus) were added to wells 1 – 9, subsequently, the M_1dG levels derived were used to determine the calibration curve. DNA samples added to wells 10 – 24 were derived from human prostate cancer cells and murine xenograft tissues from the experiments described in chapters 3 and 4.

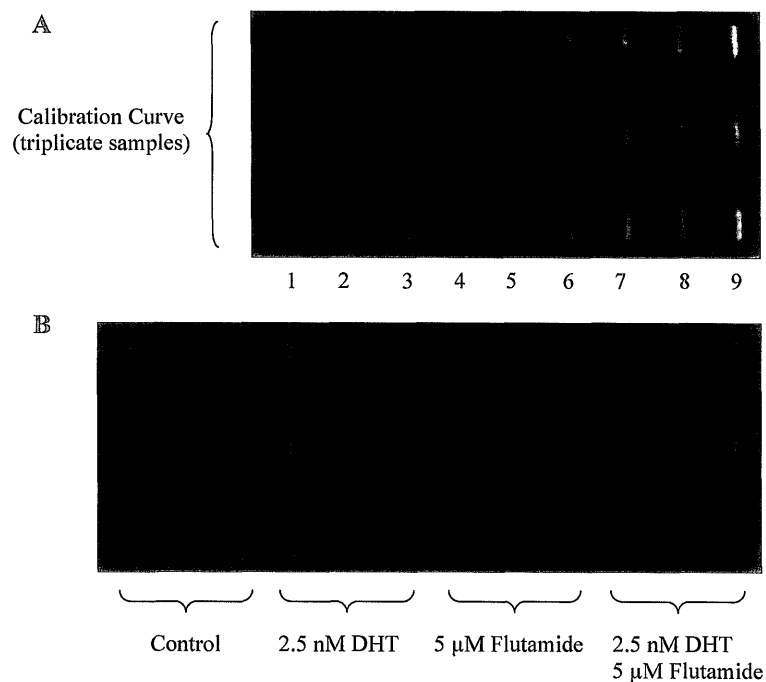


Figure 2.3.3.3 Digital bioimage of immunoslot blot used to determine M_1dG levels. The addition of chemiluminescent reagents to the nitrocellulose filter paper and then exposure of the paper on the genegnome bioimaging system generated the digital image. Each band was quantified and the mean value was calculated from the triplicate bands of the individual columns. Columns 1 – 9 represent the serial samples of calf thymus DNA (A) and were used to determine the calibration curve (Figure 2.3.3.4). Columns (B) represent samples of DNA extracted from human prostate cancer cells (LNCaP) that were treated with vehicle-control, dihydrotestosterone (DHT), flutamide and a combination of flutamide and DHT. Each experiment was carried out in triplicate.

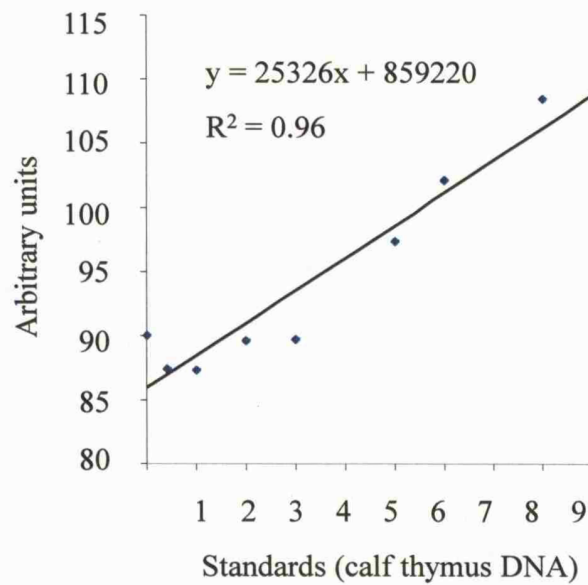


Figure 2.3.3.4 Calibration curve used to determine M_1dG levels. The calibration curve was produced by the values obtained from the mean of the triplicate bands in each column 1 – 9 (Figure 2.3.3.3). M_1dG levels were then calculated for the DNA samples in columns 10 – 24 using the calibration curve. The R^2 value represents a good linear relationship.

2.3.4 DNA extraction from cells for the determination of 8-oxo-dG levels

DNA extraction from human prostate cancer cells was performed for 8-oxo-dG analysis using the protocol based on the European Standards Committee on Oxidative DNA Damage [ESCODD] (Collins, 2002).

- Cells were harvested from T175cm² using 10 mls of trypsin (1%).
- Cell suspensions were centrifuged in 50 ml falcon tubes containing 40 mls of cell media, at 500 x g for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was re-suspended initially in 2 mls of cell media followed by further dilution in 5-8 mls of cell media.
- Cell counts were performed using trypan blue and the haemocytometer.
- Ice cold PBS was added to the cell suspension to make up to a final volume of 50 mls. The suspension was centrifuged at 700 x g for 7 minutes at 4°C. The supernatant was discarded and the cell pellet was again re-suspended in 2 mls of PBS, followed by 48 mls of PBS and repeat centrifugation.
- Supernatant was discarded and the cell pellet was re-suspended in 1.5 mls of ice cold Buffer A, then transferred to a 2 ml eppendorf tube.
- Centrifugation of nuclei at 1500 x g for 5 minutes at 4°C.
- Supernatant discarded and 1.5 mls of Buffer A added to the nuclear pellet, sample vortexed for 10 seconds.
- Centrifugation of nuclei at 1500 x g for 5 minutes at 4°C.
- Supernatant discarded and 600 µl of Buffer B was added to the pellet. Vortexed until the pellet was well dispersed. Then 35 µl of 10% SDS was added and vortexed again.

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- RNAase IIIA (3 μ ls) and RNAase T1 (8 μ ls) were added, followed by gentle vortex for 10 seconds.
- Incubation at 50°C in water bath for 15 minutes.
- Allowed to cool and protease (30 μ ls) added. Gentle vortex for 10 seconds.
- Incubation at 37°C in water bath for 1 hour.
- Allowed to cool then contents transferred to a 15 ml falcon tube containing 1.2 mls of sodium iodide solution. Vortexed vigorously for 30 seconds.
- 2mls of isopropanol (100%) was added, followed by gentle inversion of the tube several times to mix.
- Centrifugation at 5000 x *g* for 5 minutes at 20°C.
- Supernatant discarded and DNA pellet washed with 1-2 mls of isopropanol (40%). Centrifugation as above.
- Supernatant discarded and DNA pellet washed with 1-2 mls of ethanol (70% at -20°C). Centrifugation as above but at 4°C.
- Ethanol completely removed and pellet allowed to partially dry at room temperature for 5 minutes.
- The DNA pellet was suspended in 200 μ ls of Desferoxamine mesylate (0.1 mM) and stored at -80°C.

Ultra-violet spectrophotometry previously described in section 2.3.1 was used to calculate the volume of DNA sample required to give 50 µg of DNA, which was, subsequently, used in the determination of 8-oxo-dG levels.

2.3.5 Determination of 8-oxo-dG levels in DNA samples using immunoaffinity column purification and liquid chromatography-mass spectrometry method for the determination of 8-oxo-dG.

8-oxo-dG was determined by the method described below by Singh *et al.* (2003).

Enzyme digestion of DNA

- 50 µg of DNA and 50 µl of the stable isotope internal standard – [¹⁵N₅] 8-oxo-dG (1pmol/µL) was added into a 1 ml eppendorf, vortexed and centrifuged to dryness using the DNA speed vac (no heat).
- 30 µl of digestion buffer was added, followed by 15.5 µl of snake venom phosphodiesterase (SVP), 2.5 µl deoxyribonuclease (DNAase) and 2.0 µl of shrimp alkaline phosphatase (SAP).
- Incubated at 37°C in water bath for 2 hours.
- Centrifugation at 14,000 rpm for 5 minutes. 5 µl of the digested DNA was aliquoted for HPLC-UV to determine the level of 2'-deoxyguanosine.
- Remaining 45 µl digested DNA transferred to a microcon centrifugal filter device (Cut off 10,000 MW) and centrifuged at 14,000 rpm for 1 hour at 4°C.
- Discarded filter device, the remaining sample in solution was stored at (-20°C).

Elution of DNA from the immunoaffinity column

- Filtered digested DNA samples were diluted with loading buffer (0.05M sodium phosphate, 0.02% sodium azide (g/v), pH 7.4) to a final volume of 1.0 ml
- Apply solution to immunoaffinity column. The column was allowed to elute by gravity at room temperature.
- Elute unbound material using 5.0 mls of loading buffer followed by 5.0 mls of HPLC grade water.
- Elute 8-oxo-dG using 5.0 mls of 50% methanol/water (v/v) into a 15 ml falcon tube.
- Regenerate the column for subsequent sample with 5.0 mls of loading buffer followed by 5.0 mls of HPLC grade water.
- The 50% methanol/water fraction was evaporated to 1.0 ml using the DNA speed vac; the fraction was then transferred to a 2 ml eppendorf and evaporated to complete dryness using the DNA speed vac.

2.3.6 Liquid chromatography-mass spectrometry/mass spectrometry

The LC-MS/MS consisted of a Waters Alliance 2695 separations module with a 100 µl injection loop connected to a Micromass Quattro Ultima Pt. (Micromass, Waters Ltd., Manchester, UK) tandem quadruple mass spectrometer with an electrospray interface. The temperature of the electrospray source was maintained at 110°C and the desolvation temperature at 350°C. Nitrogen gas was used as the desolvation gas (650 L/h) and the cone gas was set to zero. The capillary voltage was set at 3.20 kV. The cone and RF1 lens voltages were 42 V and 30 V,

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respectively. The collision gas was argon (indicated cell pressure 2.0×10^{-3} mbar) and the collision energy set at 12 eV. The dwell time was set to 200 ms and the resolution was one m/z unit at peak base. The samples were analysed in positive electrospray ionization MS/MS selected reaction monitoring (SRM) mode for the $[M+H]^+$ ion to base $[B+H_2]^+$ transitions of 8-oxodG (m/z 284 to 168) and $[^{15}N_5]$ 8-oxo-dG (m/z 289 to 173). The level of 8-oxodG was determined in each sample from the ratio of the peak area of 8-oxodG to that of the internal standard $[^{15}N_5]$ 8-oxodG and normalised to the amount of 2'-deoxyguanosine observed for each sample following DNA digestion as determined by HPLC-UV (Figure 2.3.6.1).

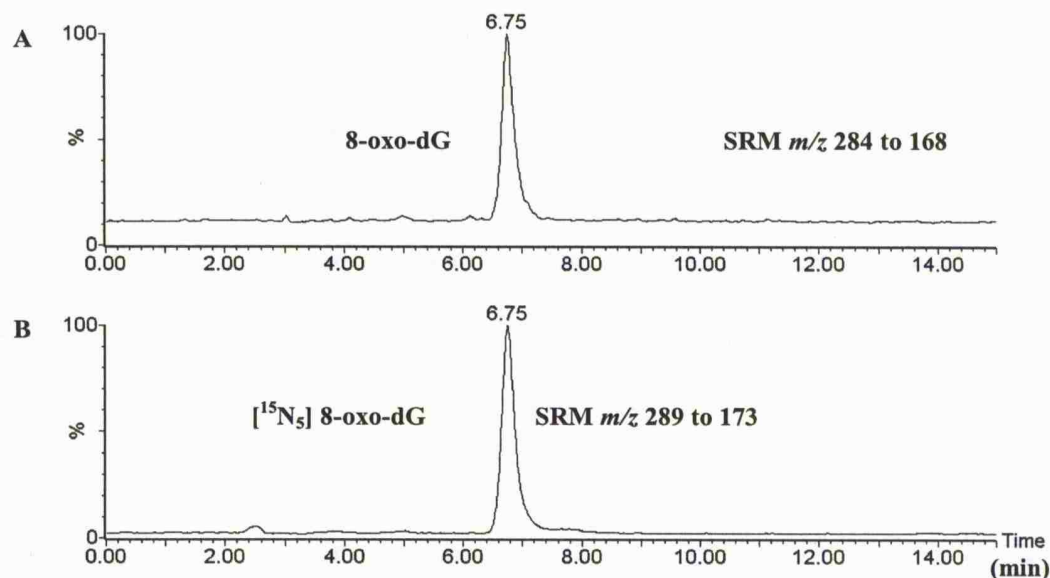


Figure 2.3.6.1 Determination of 8-oxo-dG by immunoaffinity column purification and LC-MS/MS. The above chromatograms represent one sample of DNA used to determine the level of 8-oxo-dG. The DNA was extracted from a control sample of human prostate cancer cells; LNCaP treated with 100 nM dihydrotestosterone (DHT) for 7 days. Experiments were carried out in triplicate. The level of 8-oxo-dG was determined from the ratio of the peak area of 8-oxo-dG (A) to that of the internal standard [¹⁵N₅] 8-oxo-dG (B) and normalised to the amount of 2'-deoxyguanosine observed for each sample following DNA digestion as determined by HPLC-UV.

2.4 *In Vivo* Methods

2.4.1 Effects of flutamide on M₁dG and serum PSA levels in the athymic nude mouse LNCaP xenograft.

Experiments were carried out under animal project licence PPL 40/2496 granted to the University of Leicester by the UK Home Office. The experimental design was vetted by the University of Leicester Local Ethical Committee for Animal Experimentation and met the standards set by the revised guidelines from the UK Coordinating Committee on Cancer Research (UKCCR, London, UK).

We had encountered some difficulties in creating an androgen-sensitive, LNCaP xenograft. Earlier attempts by a member of the group had failed to create the xenograft. In these experiments, we attempted to create the xenograft but this time using Matrigel® Basement Membrane Matrix. Therefore, 4 nude mice and 4 nude rats were inoculated using a 21 gauge needle with LNCaP cells (2×10^6) in matrigel. The rationale for the use of rats was to allow measurements in addition to the M₁dG levels of 8-oxo-dG levels in blood. Five mls of rat blood compared to 1.0-1.5 mls from the mice would have allowed sufficient DNA to be extracted for this to be achieved. Unfortunately, there was no evidence of tumour development in the nude rats, however, tumours developed in 3/4 of the nude mice. Tumours reached average diameters of 3 mm at 10 weeks. Ideally, we needed the tumours to be larger and to have developed in a shorter timeframe. Dr Helen McCarthy (Queens University, Belfast) had encountered similar problems but managed to successfully resolve the problems by firstly, increasing the number of LNCaP cells used to inoculate the mice with 5×10^6 / mouse; secondly,

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by using a larger bore needle, 19 gauge during subcutaneous inoculations to minimise lysis of LNCaP cells.

40 male MF-1 outbred nude mice (body weight ~ 30-40 g) were used. The mice were allowed to acclimatize for 7 days during which they were ear punched for identification purposes, randomised and were housed in Moredun isolators, with a 12 hour light-dark cycle, a temperature range of 20-23°C and 40-60% humidity. The mice were provided *ad libitum* with a high protein diet in the form of RM3 pellets and sterilised water and were maintained on irradiated bedding.

Following 7 days acclimatization, mice were divided into four groups. Group A (mice not injected with LNCaP cells and acted as non-tumour bearing controls, n=5). Group B (mice injected with LNCaP cells and were treated with vehicle control, n=12), Group C (mice injected with LNCaP cells and were treated with flutamide, n=12). Group D (mice injected with LNCaP cells but did not develop tumours and serum PSA was undetectable, and therefore, acted as non-tumour bearing, matrigel controls, n=9). Mice in Groups B, C and D were injected subcutaneously into the right flank with LNCaP cells (5×10^6) suspended in 200 μ l of Matrigel / cell culture medium. Initially, the LNCaP cells had been grown in T175cm² flasks, cells were then harvested (section 2.2.2). Using cell culture media the final cell concentration determined was $5 \times 10^6 / 100 \mu$ l. Matrigel was thawed overnight in the cold room at 4 °C and was added in equal volumes to the above, gently mixed and kept on ice prior to subcutaneous inoculation. Mice were weighed and assessed for tumour growth on a weekly basis, the latter with callipers, measuring the length and width of the tumours. The rate of tumour

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growth varied and, therefore, the experiments were carried in 3 separate batches. When the tumours reached average diameters of 5-7 mm, paired mice were randomized into Groups B and C. In all groups, pre-treatment serum PSA levels were measured from 100 μ l of tail vein blood (PSA measurements carried out by Dr Paul Garrick, Department of Biochemistry, University Hospitals of Leicester NHS Trust). Mice in group B were treated by once daily gavage with flutamide (50 mg/kg) and mice in groups A, C and D received the vehicle-control for 7 days (Luo *et al*, 1997). At the end of day 7, the experiments were terminated in all four groups by cardiac exsanguinations under light anaesthesia with halothane. Serum was separated from the blood following centrifugation at 1000 x g for 1 minute and post-treatment serum PSA levels were determined. The remaining blood was stored at -80°C and subsequently used to determine M₁dG levels (Figure 2.4.1.1). For the extraction of DNA and the determination of M₁dG see section 2.3.1 – 2.3.3. Xenograft LNCaP tumours were dissected free from mice in groups B and C. Murine prostate glands were dissected free in mice of all groups (the dissection was carried out in a saline water bath, thus allowing the prostate gland to be dissected easily from the seminal vesicles), weighed, wrapped in silver foil and snap-frozen in liquid nitrogen and stored at -80°C.

A selected number of xenograft LNCaP tumours were fixed in formalin for a minimum of 2 weeks and then embedded in paraffin wax. Sections of 5 μ m were cut and stained with haematoxylin and eosin before microscopic examination, carried out by Dr Peter Greaves (Department of Pathology, University of Leicester). The Qiagen method was used to extract DNA from the prostate glands and subcutaneous LNCaP tumours; in the latter, slices were taken from actual

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tumour avoiding necrotic areas (Figure 2.4.1.2 and 2.4.1.3). For the extraction of DNA and the determination of M₁dG see section 2.3.1 – 2.3.3.

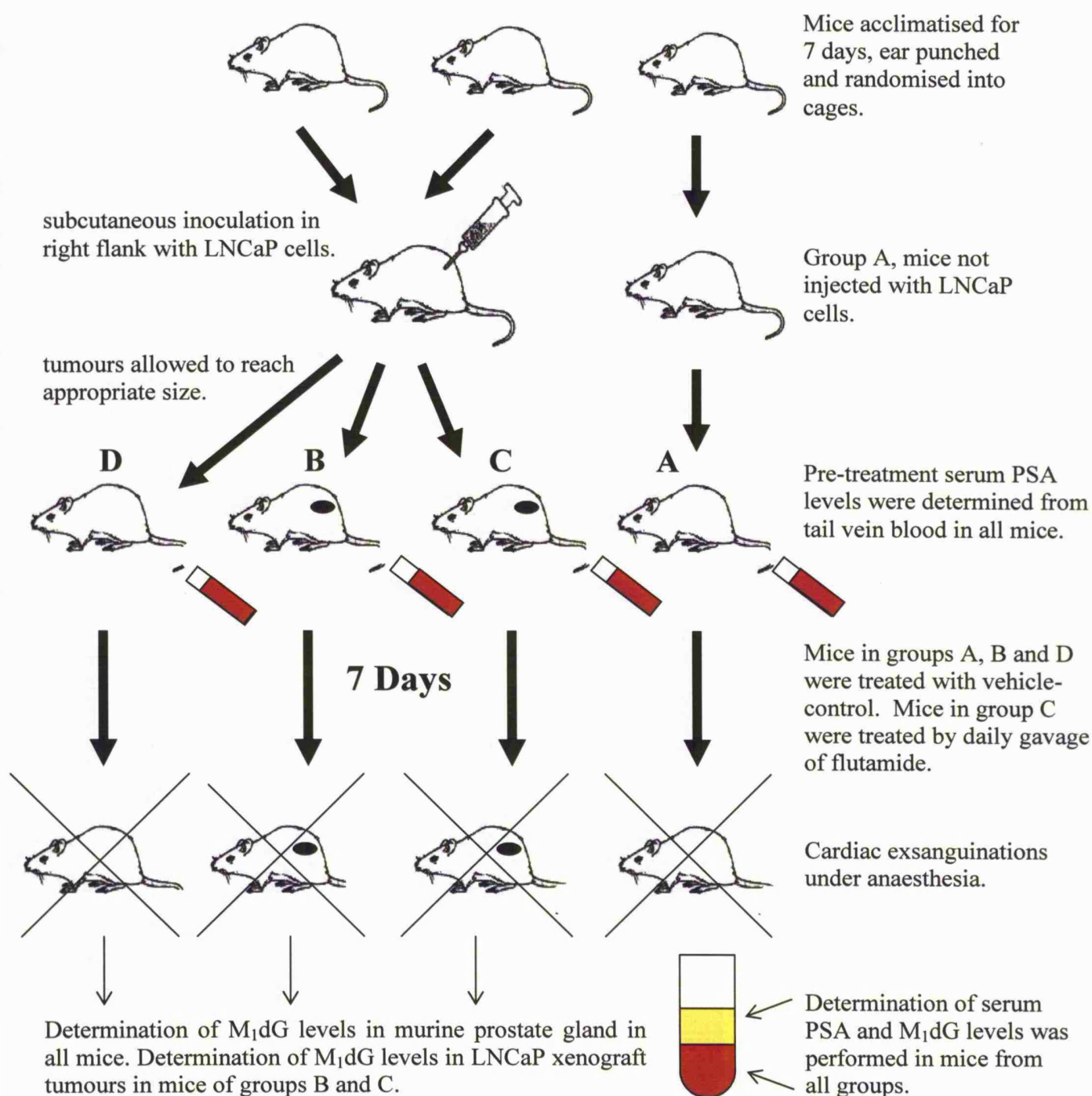


FIGURE 2.4.1.1 Effect of flutamide on M₁dG and serum PSA levels in the athymic nude mouse LNCaP xenograft. The figure illustrates the steps of the experimental design and methods used to determine the M₁dG and serum PSA levels in mice treated with flutamide (50 mg/kg *ig*) and vehicle-control daily for 7 days. A-D represent the four groups of mice, Group A (non-LNCaP injected controls), Group B (LNCaP tumour-bearing controls), Group C (LNCaP tumour-bearing, flutamide treated) and Group D (LNCaP/Matrigel injected, non tumour-bearing controls).

Figure 2.4.1.2 Pathological features of xenograft LNCaP tumours from athymic nude mice. Macroscopic appearance of tumours from mice treated with flutamide (50 mg/kg *ig*) (A) and vehicle-control (B) daily for 7 days. Photomicrographs (haematoxylin and eosin stain) represent gross necrotic areas (C), focal necrosis (D) and no necrosis in tumour tissue (E) as represented with arrows, respectively (magnification x 100, x 250 and x 250)

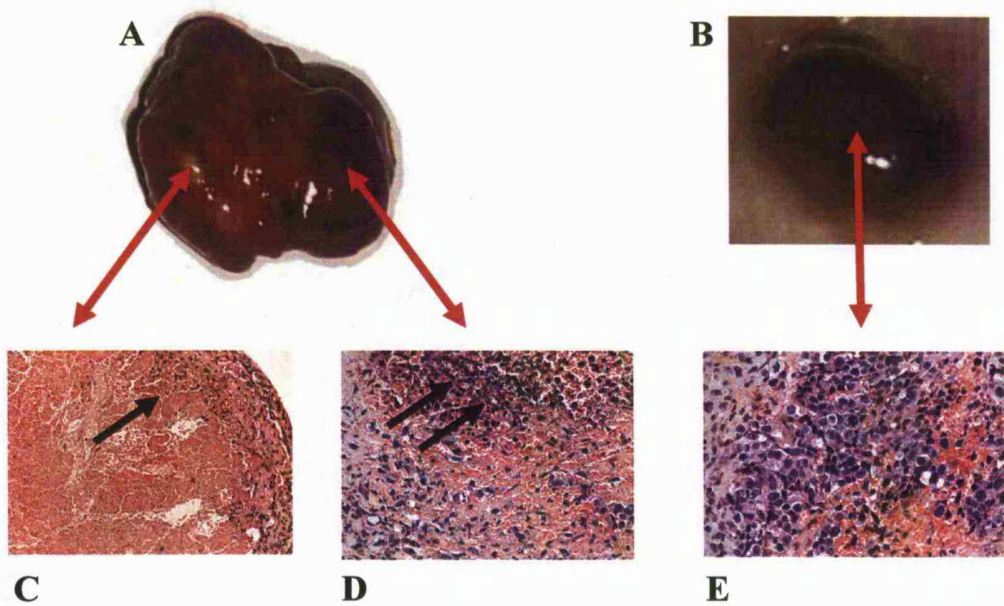
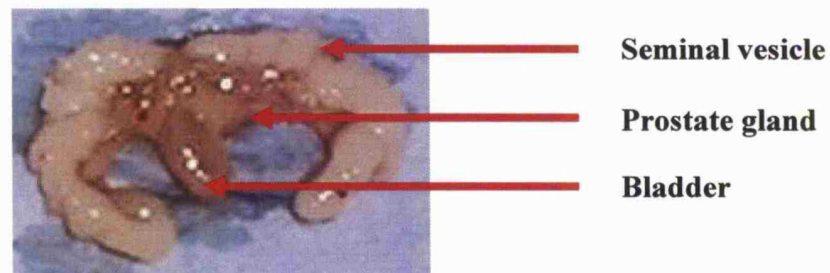


Figure 2.4.1.3 Features of a typical seminal vesicle, bladder and prostate gland from an athymic nude mouse.



2.4.2 Determination of tumour volume.

The volume of the tumour was determined by the formula (Overholser *et al*, 2000).

$$\text{Volume (mm}^3\text{)} = \pi / 6 (\text{L}_1 \times \text{L}_2 \times \text{L}_2)$$

L₁ = longest diameter (mm) and L₂ = shorter diameter (mm)

2.5 Statistical Methods

Results were subjected to analyses of variance (ANOVA) and covariance with 5% significance level using Minitab software version 14 (Minitab Inc, PA, USA). Plots of the residuals were used to ensure that variances were homogeneous and that the residuals followed a normal distribution. Non-parametric data were analysed using the Mann-Whitney test.

CHAPTER THREE

Effect of dihydrotestosterone and flutamide on oxidative DNA damage in human prostate cancer cells

3.1 Introduction

ROS are known to be factors of importance in the development and progression of prostate cancer (see section 1.3). They may cause peroxidation of membrane lipids and oxidative DNA damage, resulting in the formation of oxidative DNA adducts, which have been associated with carcinogenesis. It has been shown that androgens increase ROS in human prostate cancer cells cultured *in vitro*. However, such species are unstable, short-lived and technically difficult to measure without artefact. The purpose of this study was to determine the effect of DHT-induced ROS on oxidative DNA damage, through the formation of oxidative DNA adducts, namely, M₁dG and 8-oxo-dG in human prostate cancer cells. These adducts are known to be more stable and technically easier to measure than ROS.

The aim of the initial studies described in this chapter was to determine the cell proliferation characteristics of human prostate cancer cells, LNCaP, with DHT stimulation *in vitro*. A further aim was to determine the inhibitory effects of the competitive anti-androgen receptor antagonist, flutamide, on cell proliferation of LNCaP cells *in vitro*.

Finally, the studies described here were to determine the effect of DHT and flutamide on oxidative DNA adduct levels in both androgen-insensitive (DU145, PC3) and androgen-sensitive (LNCaP) human prostate cancer cells. Mechanistically, DHT-induced ROS and, therefore, oxidative DNA levels in LNCaP cells is mediated via the androgen receptor. ROS-induced oxidative DNA damage may also occur directly, independent of the androgen receptor.

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Therefore, we aimed to assess the direct effect of ROS on oxidative DNA damage in LNCaP cells. The agent used for this was bleomycin, a chemotherapeutic agent known to be a potent inducer of ROS and DNA damage.

The hypotheses tested in the androgen-sensitive (LNCaP) and androgen-insensitive (DU145, PC3) human prostate cancer cells were as follows:

- DHT increases M₁dG and 8-oxo-dG levels in LNCaP cells.
- Flutamide prevents the increase in M₁dG and 8-oxo-dG levels in LNCaP cells.
- Bleomycin increases M₁dG levels in LNCaP cells directly, independent of the androgen receptor.

3.2 Effect of DHT and flutamide on LNCaP cell proliferation

The stimulatory effect of DHT on cell proliferation in the androgen-sensitive, LNCaP human prostate cancer cell was determined. The inhibitory effect of flutamide, a competitive anti-androgen receptor antagonist was also determined. The biological properties of human prostate cancer cell lines have been described in Section 1.4.1.

Cells were grown in 10% FCS and treated daily with or without DHT (1-100 nM) and flutamide (1-10 μ M) for up to 168 hours. Cells were seeded at 1×10^4 per cell as described in Section 2.2.3-2.2.4. Figures 3.2.1 and 3.2.2 show the effect on growth of LNCaP cells of DHT and flutamide, respectively. Figure 3.2.3 shows the effect on growth in LNCaP cells of both flutamide and DHT in combination at varying concentrations.

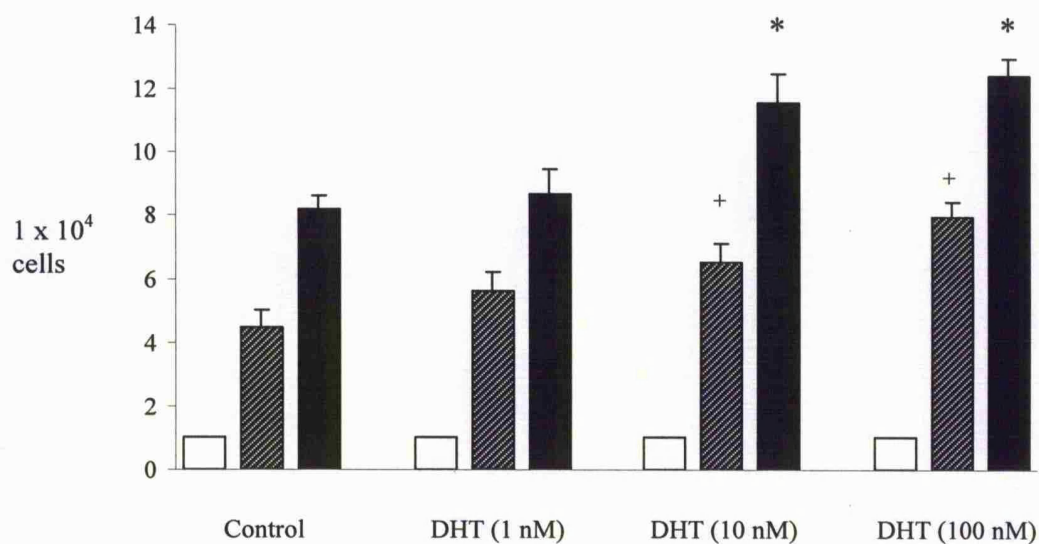


Figure 3.2.1 Effect of DHT (1-100 nM) on growth of LNCaP cells.

Represented above are the growth characteristics of LNCaP cells treated with dihydrotestosterone (DHT). LNCaP cells were grown in the presence of 10% foetal calf serum (FCS) and treated daily for 7 days with vehicle-control or DHT (1, 10 and 100 nM) as detailed in Section 2.2.3. Cell counts were performed using the coulter Z2 counter at 96 hours (hatched bars) and 168 hours (closed bars). (+) and (*) denote that the difference was significant over controls at 96 hours and 168 hours, respectively, ($p < 0.01$, student *t*-test). Values are cell numbers \pm SD. Experiments were carried out in triplicate.

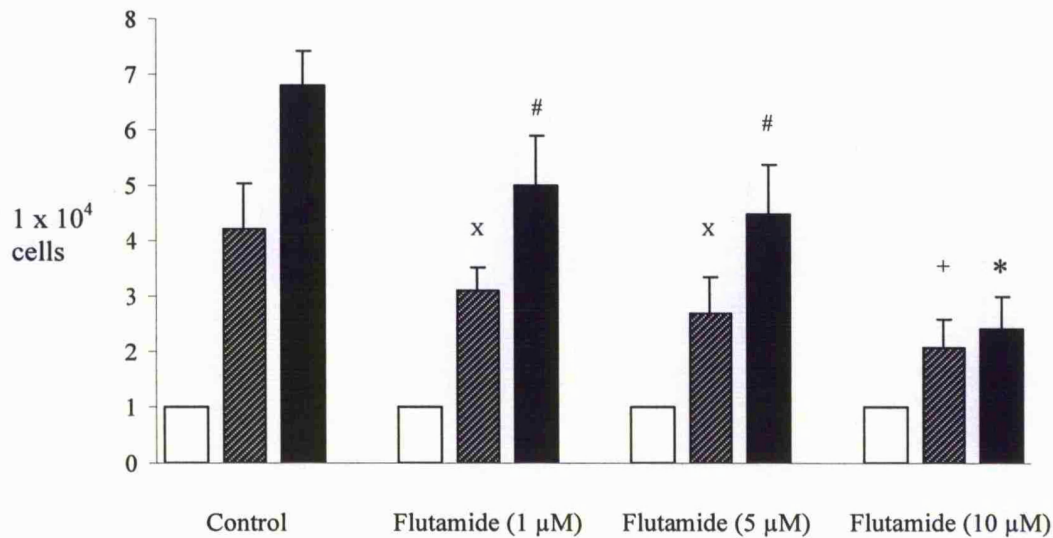


Figure 3.2.2 Effect of Flutamide (1-10 μM) on growth of LNCaP cells.

Represented above are the growth characteristics of LNCaP cells treated with flutamide. LNCaP cells were grown in the presence of 10% foetal calf serum (FCS) and treated daily for 7 days with vehicle-control or flutamide 1, 5 and 10 μM as detailed in Section 2.2.3. Cell counts were performed using the coulter Z2 counter at 96 hours (hatched bars) and 168 hours (closed bars). (+) and (*) denote that the difference was significant over controls at 96 hours and 168 hours, respectively, ($p < 0.01$, student t -test). (x) and (#) denote that the difference was significant over controls at 96 hours and 168 hours, respectively, ($p < 0.05$, student t -test). Values are cell numbers \pm SD. Experiments were carried out in triplicate.

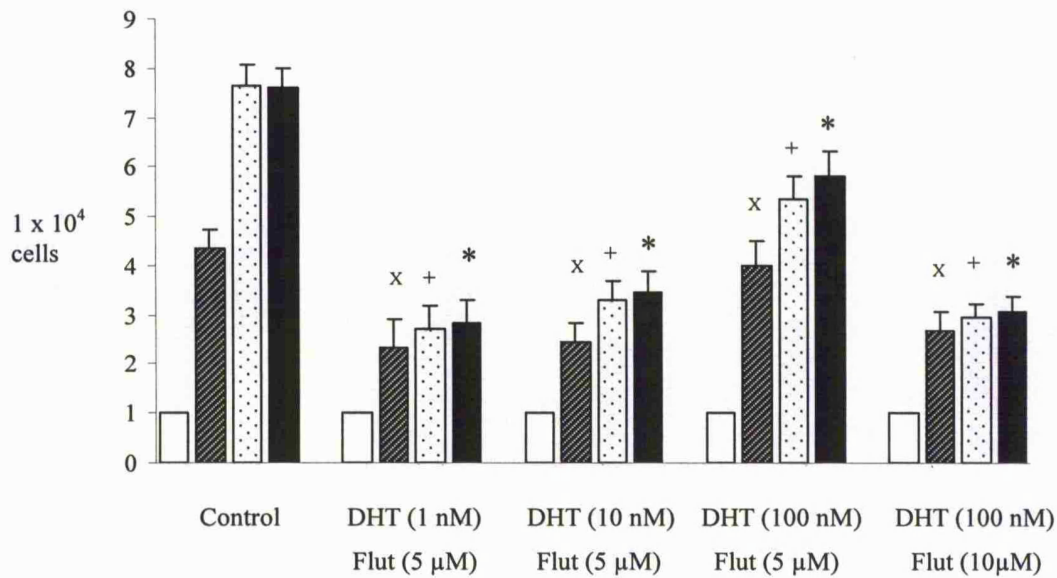


Figure 3.2.3 Effect of Flutamide (1-10 μM) and DHT (1-100 nM) in combination on growth of LNCaP cells. Represented above are the growth characteristics of LNCaP cells treated with combination of dihydrotestosterone (DHT) and Flutamide (Flut). LNCaP cells were grown in the presence of 10% foetal calf serum (FCS) and treated daily for 7 days with vehicle-control or DHT/Flut combinations as detailed in Section 2.2.3. Cell counts were performed using the coulter Z2 counter at 96 hours (hatched bars), 144 hours (dotted bars) and 168 hours (closed bars). (x), (+) and (*) denote that the difference was significant over controls at 96 hours, 144 hours and 168 hours, respectively, ($p < 0.01$, student *t*-test). Values are cell numbers \pm SD. Experiments were carried out in triplicate.

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Cell counts in Figures 3.2.1-3.2.3 are represented as $n \pm SD$ ($\times 10^4$). Figure 3.2.1 shows the effect of DHT on the growth of LNCaP cells. Cell counts for control and DHT treated 1nM, 10 nM and 100 nM at 96 and 168 hours were 4.4 ± 0.6 , 8.2 ± 0.4 , 5.6 ± 0.6 , 8.7 ± 0.8 , 6.5 ± 0.6 , 11.8 ± 0.9 , 8.0 ± 0.4 and 12.5 ± 0.5 , respectively. Growth of LNCaP cells treated with 10 nM and 100 nM DHT was statistically significant over controls at 96 and 168 hours, respectively, ($p < 0.01$, student *t*-test).

Figure 3.2.2 shows the effect of co-administration of flutamide and DHT on the growth of LNCaP cells. Cell counts for control and flutamide treated 1 μ M, 5 μ M and 10 μ M at 96 and 168 hours were 4.2 ± 0.8 , 6.8 ± 0.6 , 3.1 ± 0.4 , 5.0 ± 0.9 , 2.7 ± 0.6 , 4.5 ± 0.9 , 2.0 ± 0.5 and 2.4 ± 0.6 , respectively. Growth inhibition of LNCaP cells treated with flutamide at 1 μ M and 5 μ M was statistically significant over controls at 96 and 168 hours, respectively, ($p < 0.05$, student *t*-test) and at the corresponding time points with 10 μ M flutamide ($p < 0.01$, student *t*-test).

Figure 3.2.3 shows the effect of flutamide on the growth inhibition of LNCaP cells. Cell counts for control and flutamide + DHT treated 5 μ M + 1 nM, 5 μ M + 10 nM, 5 μ M + 100 nM and 10 μ M + 100 nM at 96, 144 and 168 hours were 4.3 ± 0.4 , 7.7 ± 0.4 , 7.6 ± 0.4 , 2.3 ± 0.6 , 2.7 ± 0.7 , 2.8 ± 0.5 , 2.5 ± 0.4 , 3.3 ± 0.4 , 3.5 ± 0.4 , 4.0 ± 0.5 , 5.4 ± 0.5 , 5.8 ± 0.5 , 2.7 ± 0.4 , 2.9 ± 0.3 and 3.1 ± 0.3 , respectively. Growth inhibition of LNCaP cells treated with a combination of flutamide and DHT was statistically significant over controls with all combination concentrations at 96, 144 and 168 hours, respectively, ($p < 0.01$, student *t*-test).

3.3 Effect of DHT on levels of M₁dG and 8-oxo-dG in human prostate cancer cells

The effect of supra-physiological levels of DHT on levels of M₁dG and 8-oxo-dG in human prostate cancer cells was tested. DU145, PC3 and LNCaP cells were grown in 10% FCS and treated daily with DHT (100 nM) or vehicle-control for 7 days as described in section 2.2.4. The rationale for daily treatment for 7 days were based firstly, on the relatively slow growth of LNCaP cells and secondly, M₁dG had previously been shown to be elevated in human colon cells grown *in vitro* (Sharma *et al.*, 2001). The cells were harvested and DNA extracted. M₁dG and 8-oxo-dG levels were determined as described in section 2.3.

Figure 3.3.1 shows the effect of DHT (100 nM) on mean levels of M₁dG and 8-oxo-dG in human prostate cancer cells. M₁dG levels in DU145, PC3 and LNCaP cells not exposed to DHT were 7.5 ± 6.8 , 6.9 ± 4.7 and 14.0 ± 3.6 adducts/ 10^7 nucleotides, respectively. M₁dG levels in DHT-treated cells were 7.4 ± 4.7 , 7.9 ± 5.0 and 20.4 ± 4.5 adducts/ 10^7 nucleotides, respectively. 8-oxo-dG levels in DU145, PC3 and LNCaP cells not exposed to DHT were 4.4 ± 1.3 , 2.8 ± 0.4 and 2.7 ± 0.9 adducts/ 10^6 nucleotides, respectively. 8-oxo-dG levels in DHT-treated cells were 4.2 ± 1.2 , 3.6 ± 0.5 and 5.7 ± 1.2 adducts/ 10^6 nucleotides, respectively. The difference in adduct levels between control and cells exposed to supra-physiological levels of DHT was significant only for M₁dG and 8-oxo-dG levels in LNCaP cells ($P < 0.05$ by ANOVA). In contrast, such an increase was not observed in the androgen-insensitive DU145 and PC3 cells.

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To explore the significant findings of increased levels of oxidative DNA adducts in LNCaP cells, further experiments were carried out to determine a concentration-dependent response. LNCaP cells were grown in 10% FCS and treated daily with DHT (2.5-100 nM) or vehicle-control for 7 days as described in section 2.2.4. The cells were harvested and DNA extracted. M₁dG and 8-oxo-dG levels were determined as described in section 2.3.

Figure 3.3.2 shows the concentration-dependent effect of DHT on levels of M₁dG and 8-oxo-dG. M₁dG and 8-oxo-dG levels in controls and DHT treated cells (2.5, 10, 25, 50 and 100 nM) were 11.4 ± 0.7 , 12.2 ± 0.5 , 10.6 ± 3.2 , 22.1 ± 6.4 , 23.5 ± 3.6 , 24.1 ± 7.6 adducts/ 10^7 nucleotides and 2.2 ± 0.5 , 2.9 ± 0.3 , 3.1 ± 0.2 , 4.9 ± 1.2 , 5.8 ± 1.8 , 6.2 ± 1.7 adducts/ 10^6 nucleotides, respectively. Both oxidative DNA adducts were significantly elevated in LNCaP cells over control levels at concentrations of DHT of 25 nM ($P < 0.05$ by ANOVA).

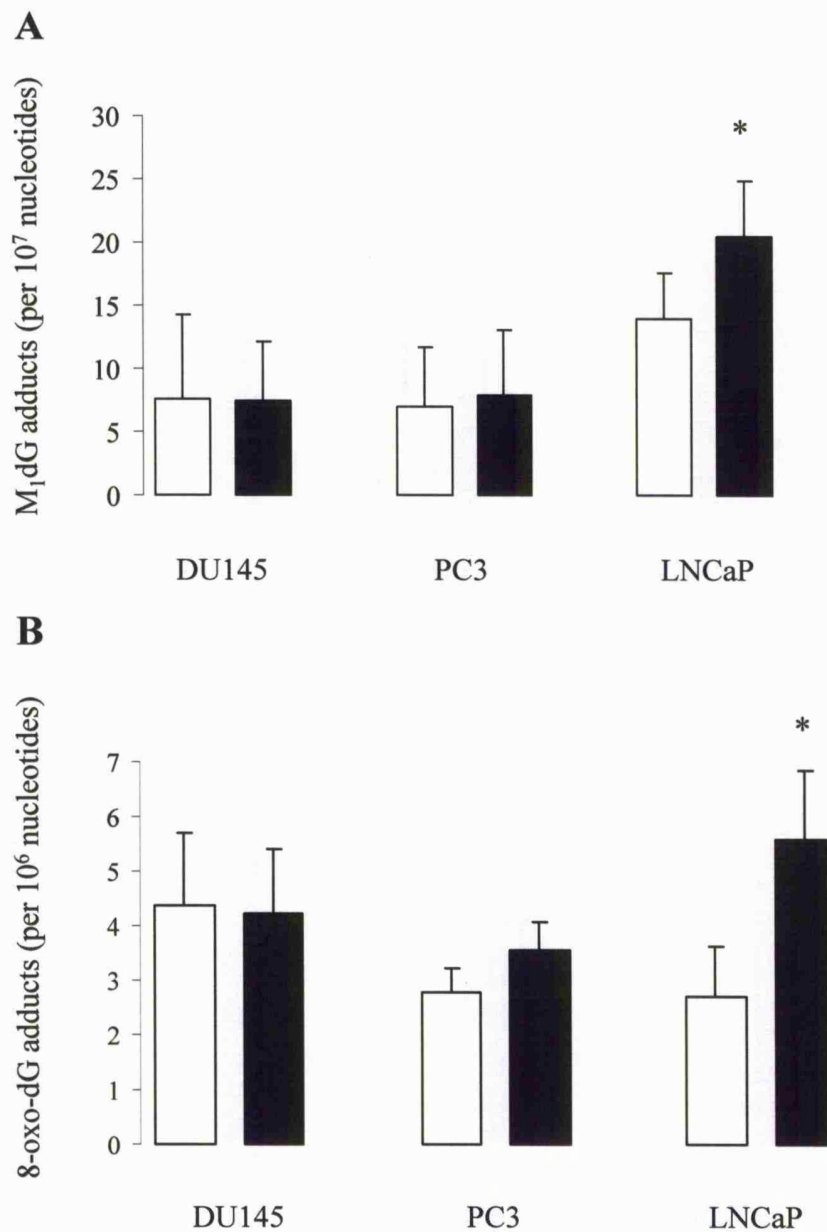


Figure 3.3.1. Effect of daily treatment with DHT (100 nM) on levels of M₁dG (A) and 8-oxo-dG (B) in DU145, PC3 and LNCaP cells. Open bars denote control cells and closed bars denote treated cells. Cells were seeded (approx 10⁶) in flasks and grown in medium containing 10% FCS. DHT was added daily for 7 days. Results (mean + SD) are presented from 3 separate experiments. * indicate that the difference is significant compared with control cells ($p < 0.05$ by ANOVA). For details of cell culture and M₁dG and 8-oxo-dG measurements see section 2.2.4 and 2.3.

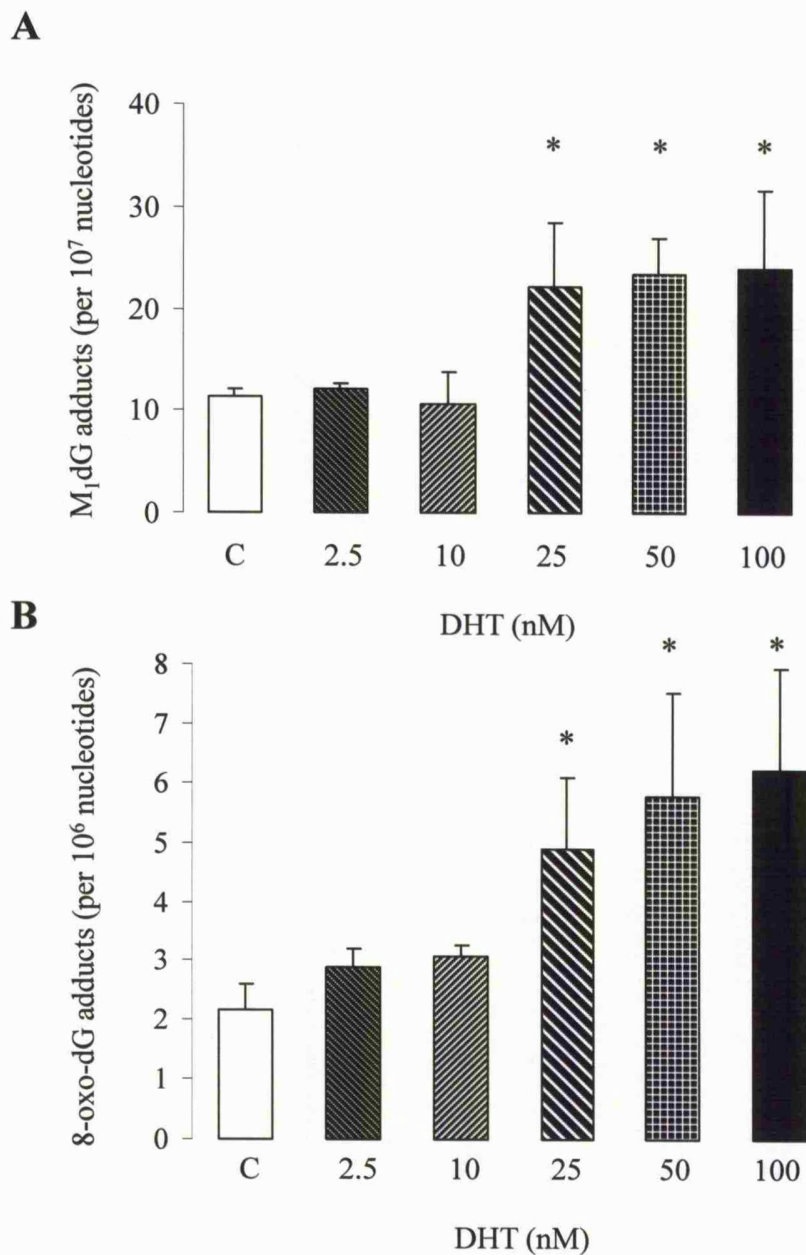


Figure 3.3.2 Concentration – dependent of effect of DHT on levels of M₁dG (A) and 8-oxo-dG (B) in LNCaP cells. Open bars denote control cells (C) and closed bars denote treated cells. Cells were seeded (approx 10⁶) in flasks and grown in medium containing 10% FCS. DHT was added daily for 7 days. Results (mean + SD) are presented from 3 separate experiments. * indicate that the difference to respective control cells is significant ($p < 0.05$ by ANOVA). For details of cell culture and M₁dG and 8-oxo-dG measurements see section 2.2.4 and 2.3.

3.4 Effect of flutamide on increase in M₁dG and 8-oxo-dG caused by DHT in LNCaP cells.

The results of the studies shown in section 3.3 revealed an increase in oxidative DNA adduct levels in LNCaP cells induced by DHT. This effect was concentration-dependent at supra-physiological levels. However, these experiments were carried out in cell culture media containing 10% FCS including the controls. Therefore, we hypothesised that there was a difference at physiological concentrations of DHT but that this may have been masked by the androgens in 10% FCS. We carried out experiments described here that were serum-free. Furthermore, we also determined the effect of flutamide in preventing increased oxidative DNA adduct levels.

LNCaP cells were grown in 10% FCS for 3 days. The cell culture media was then changed to serum-free. Cells were treated with Flutamide, DHT and the two in combination for 4 days as described in section 2.2.4. Cells were harvested and DNA extracted. M₁dG and 8-oxo-dG levels were determined as described in section 2.3.

Figure 3.4.1 shows the effect of flutamide (5 μ M) on increase in M₁dG (A) or 8-oxo-dG (B) caused by DHT (2.5 nM). M₁dG and 8-oxo-dG levels in controls, DHT (2.5 nM) treated cells, flutamide (5 μ M) treated cells and a combination of the latter two concentrations were 9.9 ± 2.3 , 15.9 ± 2.6 , 10.1 ± 1.9 , 11.8 ± 1.1 adducts/ 10^7 nucleotides and 2.8 ± 0.8 , 4.9 ± 0.6 , 2.9 ± 0.6 , 3.0 ± 0.3 adducts/ 10^6 nucleotides, respectively.

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Figure 3.4.2 shows the effect of flutamide (10 μM) on increase in M₁dG (A) or 8-oxo-dG (B) caused by DHT (100 nM). M₁dG and 8-oxo-dG levels in controls, DHT (100 nM) treated cells, flutamide (10 μM) treated cells and a combination of the latter two concentrations were 12.8 ± 2.6 , 21.3 ± 4.8 , 11.6 ± 4.2 , 13.3 ± 4.0 adducts/ 10^7 nucleotides and 2.9 ± 0.8 , 6.2 ± 1.5 , 4.0 ± 0.3 , 3.9 ± 0.2 adducts/ 10^6 nucleotides, respectively. Flutamide counteracted the increase in M₁dG and 8-oxo-dG levels by DHT at the appropriate corresponding concentrations. The results were statistically significant ($P < 0.05$ by ANOVA).

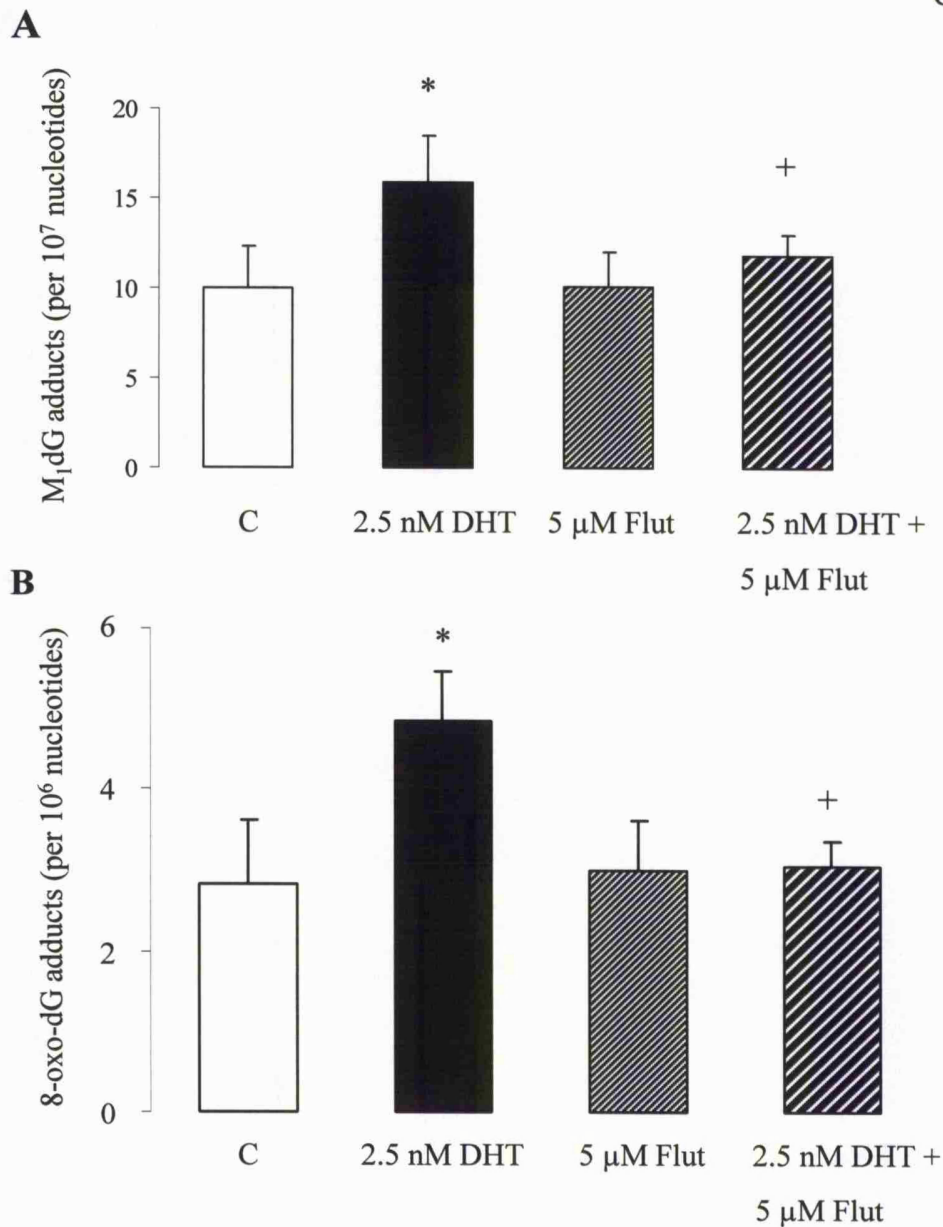


Figure 3.4.1 Effect of flutamide (5 μ M) on increase in M₁dG (A) and 8-oxo-dG (B) levels caused by DHT (2.5 nM) in LNCaP cells. Open bars denote control cells (C) and closed bars denote treated cells. Cells (approx 10⁶) were seeded in flasks and grown for 3 days in medium containing 10% FCS. Cells were subsequently grown in serum-free conditions with or without the daily addition of flutamide and/or DHT for 4 days. Results (mean + SD) are presented from 3 separate experiments. * indicate that the difference to control cells is significant, + indicate that the difference to cells incubated with DHT alone is significant ($p < 0.05$ by ANOVA). For details of cell culture and M₁dG and 8-oxo-dG measurements see section 2.2.4 and 2.3.

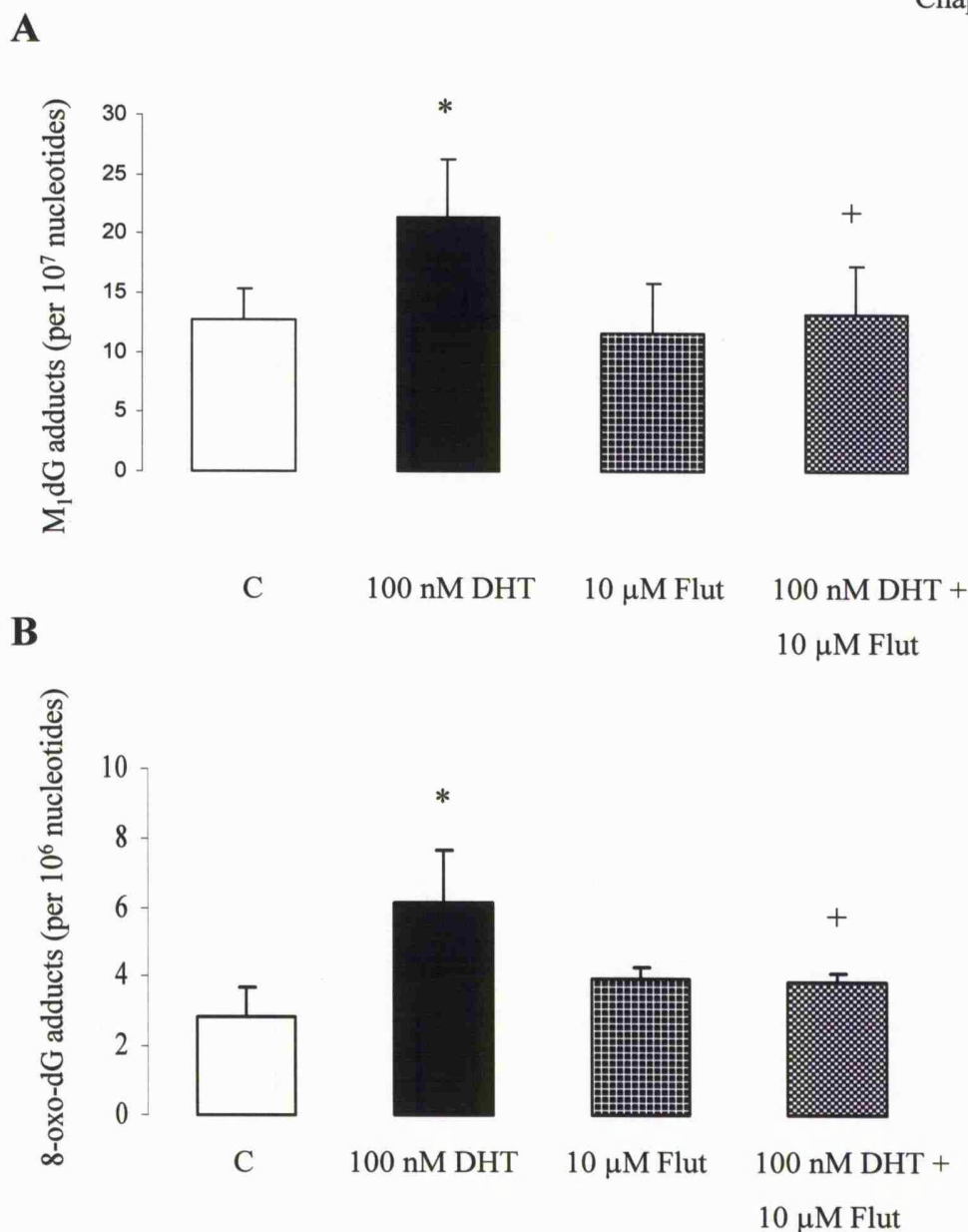


Figure 3.4.2 Effect of flutamide (10 μM) on increase in M₁dG (A) and 8-oxo-dG (B) levels caused by DHT (100 nM) in LNCaP cells. Open bars denote control cells (C) and closed bars denote treated cells. Cells (approx 10⁶) were seeded in flasks and grown for 3 days in medium containing 10% FCS. Cells were subsequently grown in serum-free conditions with or without the daily addition of flutamide and/or DHT for 4 days. Results (mean + SD) are presented from 3 separate experiments. * indicate that the difference to control cells is significant, + indicate that the difference to cells incubated with DHT alone is significant ($p < 0.05$ by ANOVA). For details of cell culture and M₁dG and 8-oxo-dG measurements see section 2.2.4 and 2.3.

3.5 Effect of bleomycin on LNCaP cell viability and M₁dG levels

Bleomycin is a chemotherapeutic agent used against a number of human cancers. Its principal mode of action is to cause oxidative DNA damage and DNA fragmentation. The results of the studies shown in section 3.3 and 3.4 revealed an increase in oxidative DNA adducts in LNCaP cells induced by DHT. The mechanism for this increase was mediated via the androgen receptor. We determined the effect of direct oxidative DNA damage induced by ROS generated by bleomycin. This aspect was considered important as it might allow insight into ROS damage independent of the DHT-androgen complex. Based on the results of this experiment, the extent of oxidative DNA damage induced by DHT would be indirectly inferred. We tested the hypothesis that bleomycin increases ROS leading to an increase in M₁dG levels.

LNCaP cells were grown and treated with bleomycin (5 μ M and 20 μ M) for 1, 2, 3, 4, 5 and 6 hours. Cells were harvested and cell viability was determined as described in section 2.2.4 and 2.2.5. Subsequently, LNCaP cells were also grown and treated with bleomycin (20 μ M) for 1, 3, 6, 15, 24 and 48 hours. The cells were harvested and DNA extracted. M₁dG levels were determined as described in section 2.3.

Cell viability in control and bleomycin (5 μ M and 20 μ M) treated LNCaP cells was 98%, 99% and 98%, respectively. Exposure to bleomycin failed to affect cell viability. Figure 3.5.1 shows the effect on M₁dG levels in LNCaP cells of varying time to bleomycin (20 μ M). The M₁dG levels in control and treated cells for 1, 3,

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6, 15, 24 and 48 hours were 11.1 ± 0.6 , 12.1 ± 2.8 , 12.4 ± 6.7 , 11.5 ± 1.9 , 10.4 ± 5.4 , 8.3 ± 4.8 and 9.0 ± 3.4 adducts/ 10^7 nucleotides, respectively. There was no significant difference between control and treated cells.

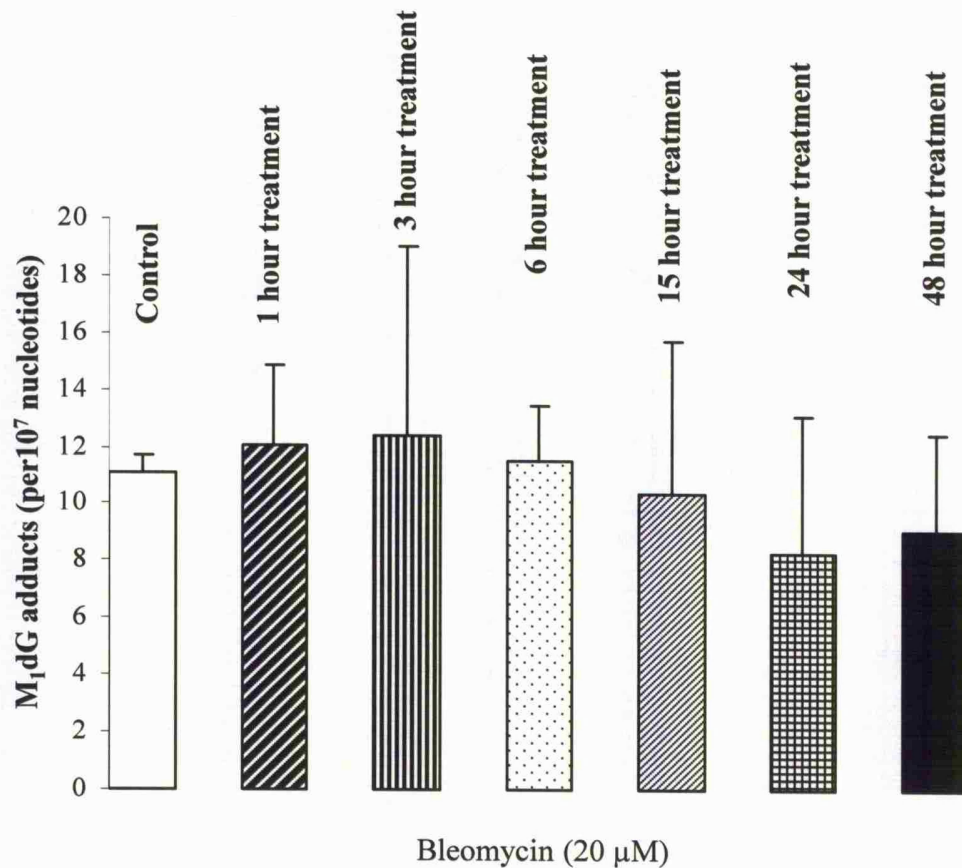


Figure 3.5.1 Effect on M₁dG levels in LNCaP cells of varying time exposure to bleomycin (20 μ M). Open bars denote control cells (C) and closed bars denote treated cells. Cells were seeded (approx 10^6) in flasks and grown in media containing 10% FCS for 5 days. Bleomycin was added for the duration (hours) as stated. Results (mean + SD) are presented from 3 different experiments. There was no significant difference between controls and treated cells.

3.6 Discussion

Figure 3.2.1 shows that the proliferation of the androgen-sensitive LNCaP cells increased with increasing concentrations of DHT; this was statistically significant over controls at 96 and 168 hours with 10 nM DHT and 100 nM DHT ($p < 0.01$, student *t*-test). Figure 3.2.2 shows that flutamide inhibited the growth of LNCaP cells; this was statistically significant over controls at 96 and 168 hours with 1 μ M and 5 μ M flutamide ($p < 0.05$, student *t*-test), and at the corresponding time points with 10 μ M ($p < 0.01$, student *t*-test). Figure 3.2.3 shows that co-administration of flutamide and DHT inhibited the growth of LNCaP cells; this was statistically significant over controls with all combination concentrations at 96, 144 and 168 hours ($p < 0.01$, student *t*-test). The results showing the effect of DHT and flutamide on LNCaP cell proliferation are consistent with the work of Bologna *et al.* (1995). Although, both flutamide at 1 μ M and 5 μ M inhibited the growth of LNCaP cells (Figure 3.2.3), similar to Sun *et al.* (2001) we chose to use the flutamide concentration of 5 μ M for our subsequent experiments.

Both M₁dG and 8-oxo-dG levels were significantly increased in LNCaP cells treated with supraphysiological levels of DHT compared with untreated controls (Figure 3.3.1). In the androgen-insensitive DU145 and PC3 cells, no difference was seen. O'Ripple *et al.* (1997) showed that LNCaP cells treated with 100 nM DHT significantly increased ROS. The method used by this group was 2', 7'-dichlorofluorescein. In this method, the dye fluoresces in the presence of hydrogen peroxide or hydroxyl radicals. Our results show for the first time that DHT induces oxidative DNA damage *in vitro*, manifest by increased levels of oxidative DNA adducts. The concentration-dependent effect was seen at concentrations \geq

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25 nM DHT. It is possible that FCS in cell culture media contained physiological concentrations of androgens that may have masked the effect of exogenous added DHT (Raivio *et al.*, 2003). Therefore, the experiments were repeated under conditions of serum deprivation. The physiological effects of DHT (2.5 nM) were seen by increased M₁dG and 8-oxo-dG levels compared with controls. Moreover, this effect was inhibited with flutamide at 5 μ M.

Following the initial work of O'Ripple *et al.* (1997) and O'Ripple *et al.* (1999), that showed a link between increased ROS induced by androgens, further work has been carried out to investigate this concept. Sun *et al.* (2001), using the 2',7'-dichlorofluorescein method, found that levels of ROS were significantly increased in a concentration-dependent manner in LNCaP cells treated with testosterone. Furthermore, this effect was prevented by co-administration of flutamide. No effect was seen in the androgen-insensitive PC3 and DU145 cells. Since testosterone stimulates the production of PSA, these workers also tested the hypothesis that the generation of ROS was PSA mediated. They treated LNCaP cells with testosterone and anti-PSA antibodies *in vitro*. Their results showed that ROS generation was blocked by anti-PSA antibodies, inferring that PSA, downstream is responsible for the oxidative stress observed. To support this theory, they exposed both DU145 and PC3 cells to PSA *in vitro*; there was a significant increase in ROS generation. These results support the theory that the effect of increased ROS observed with testosterone stimulation are not due to LNCaP cell proliferation (Liehr, 1997). Although the initial work of O'Ripple *et al.* (1997), suggested that androgens stimulated the generation of ROS in LNCaP cells

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required a functional androgen receptor, the work of Sun *et al.* (2001), clearly, suggests that the mechanism is independent of the androgen receptor.

Bleomycin is a chemotherapeutic drug used to treat a number of human cancers including lymphomas, squamous cell carcinomas and testicular tumours (Ichikawa *et al.*, 1969; Terasima and Umezawa, 1970). It exerts its effect by irreversible DNA damage mediated principally by ROS damaging and fragmenting double stranded DNA, resulting in cell death (Otteneder *et al.*, 2006; Templin *et al.*, 1992). Bleomycin increases M₁dG levels in naked calf thymus DNA when treated with 5 μ M and 20 μ M. This response was concentration-dependent (Dedon *et al.*, 1998). The effect of bleomycin on cell viability was tested (see section 3.5). The results show that there was no significant cell death seen in bleomycin treated cells. Nevertheless, the effect of bleomycin on LNCaP cells was studied because cellular DNA damage has been shown to occur in the absence of growth inhibition (Berry *et al.*, 1985; Templin *et al.*, 1992). Figure 3.5.1 shows the effect on M₁dG levels in LNCaP cells of varying time exposure to bleomycin (20 μ M). There was no significant difference in M₁dG levels between control and treated cells. One may speculate as to the reasons why no effect of bleomycin on LNCaP cells was seen. Firstly, bleomycin may have failed to penetrate the LNCaP cell membranes. This is plausible given that a number of cell lines such squamous carcinoma and HeLa cell lines display such bleomycin-resistant mechanisms (Lazo *et al.*, 1989; Urade *et al.*, 1992). Secondly, one can not exclude the possibility that bleomycin may have been inactivated by factors produced by LNCaP cells.

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The close correlation of M₁dG and 8-oxo-dG levels as shown by Kadlubar *et al.* 1998 and supported by our results from earlier experiments, it was unlikely that bleomycin would alter 8-oxo-dG levels in LNCaP cells. Therefore, these experiments were not performed.

Insulin-like growth factor works independent of the DHT-androgen receptor complex. We did use IGF-1, as an alternative to bleomycin; however, there was greater than 50% cell death within 48 hours, therefore, these experiments were abandoned (results not shown).

CHAPTER FOUR

**Effect of flutamide on oxidative DNA damage
in the athymic nude mouse LNCaP xenograft**

4.1 Introduction

The purpose of this study was to extend the results obtained in cells *in vitro* to a rodent model in which the same cancer cells (LNCaP) could be grown *in vivo*. DHT stimulated the generation of ROS in LNCaP cells *in vitro*, as reflected by increased oxidative DNA adduct levels. Furthermore, the competitive androgen receptor antagonist, flutamide prevented the effects of DHT on increasing ROS, thus, preventing oxidative DNA damage and levels of M₁dG.

The principal aim of this *in vivo* work was to test the following hypotheses:

- Flutamide inhibits the growth of LNCaP xenograft tumours.
- Flutamide reduces the levels of PSA produced by LNCaP xenograft tumours.
- Flutamide prevents the increase in M₁dG levels in LNCaP xenograft tumours.
- Flutamide prevents the increase in M₁dG levels in blood leucocytes and murine prostate gland.
- A correlation exists between M₁dG and serum PSA levels.

LNCaP cells were implanted in athymic nude mice to create an LNCaP xenograft. When tumours reached an average of 5-7 mm in size, the mice were randomised into Groups B (control) and C (treatment), receiving flutamide (50 mg/kg *ig*) and vehicle-control daily for 7 days, respectively. Mice in Groups A (mice not injected with LNCaP cells) and D (mice injected with LNCaP cells but failed to

develop tumours) received vehicle-control daily for 7 days. The design of this study is detailed in section 2.4.

4.2 LNCaP tumour development in the athymic nude mouse.

Palpable and measurable LNCaP tumours developed in 25 of the 34 mice injected with LNCaP cells. Two mice were removed from the experiment. One mouse had lost weight and was noted to have received scratch marks from other mice in the cage during the acclimatisation period. The other mouse had to be killed because the LNCaP tumour grew larger than the project licence allowed. Therefore, 24 mice were randomised into groups B and C to receive vehicle-control and flutamide, respectively. Of the initial 40 mice, 5 were not injected with LNCaP tumour cells and were used as non-tumour bearing controls (Group A). The remaining 9 mice that had been injected with LNCaP/Matrigel but did not develop tumours and in whom serum PSA levels were undetectable, these mice were treated as non-tumour bearing matrigel controls (Group D).

4.2.1 Effect of LNCaP tumour volume on serum PSA levels in the murine LNCaP xenograft.

Figure 4.2.1.1 shows the correlation between pre-treatment tumour size and serum PSA levels in the 24 mice. ($r = 0.87$, Pearson's rank correlation test). The mean pre-treatment serum PSA level was 38.5 ng/ml and ranged between 11.0 and 92.4 ng/ml.

4.2.2 Effects of flutamide on tumour volume and serum PSA levels in the murine LNCaP xenograft

The mean pre- and post-treatment serum PSA levels in the control and treated groups were 49.3 ± 28.6 , 57.4 ± 30.8 ng/ml, respectively and 30.1 ± 21.3 , 20.7 ± 14.5 ng/ml, respectively. The mean pre- and post-treatment tumour volumes in the control and treated groups were 317.1 ± 279.8 , 505.7 ± 475.6 mm³, respectively, and 305.6 ± 395.3 , 212.5 ± 271.6 mm³, respectively. Figure 4.2.2.1 shows the change in post-dosing (% of pre-dosing). The decrease in both tumour volumes and serum PSA levels was statistically significant in treated mice compared with controls ($p < 0.001$ by ANOVA).

4.2.3 Effect of flutamide on the histological appearances of LNCaP tumours in the murine LNCaP xenograft.

Macroscopically, areas of obvious necrosis were seen in treated LNCaP tumours as shown in Figure 2.4.1.2A. Microscopically, areas of focal necrosis were seen in the LNCaP tumours of treated mice compared with controls as shown in Figure 4.2.3.1.

4.2.4 Effect of flutamide on M₁dG levels in LNCaP tumours, prostate tissue and blood leucocytes in the murine LNCaP xenograft.

Figure 4.2.4.1 shows the effect of daily treatment with flutamide (50 mg/kg *ig*) for 7 days on M₁dG levels in xenograft LNCaP tumour, murine prostate tissue and blood leucocytes from non-tumour bearing mice or mice bearing LNCaP tumours. M₁dG levels in LNCaP tumours following treatment with flutamide and vehicle-control were 10.5 ± 6.9 and 20.8 ± 4.1 adduct/ 10^7 nucleotides, respectively. This difference was statistically significant ($p < 0.001$, Mann-Whitney test). M₁dG levels in murine prostate tissue and murine blood leucocytes of mice in Groups A, D, C and B were 12.9 ± 5.4 , 11.4 ± 5.6 , 12.6 ± 6.0 , 12.0 ± 5.4 and 20.4 ± 5.1 , 17.1 ± 5.2 , 18.6 ± 5.4 , 22.2 ± 4.8 , respectively. There was no observed effect of flutamide on M₁dG levels in murine prostate tissue and blood leucocytes.

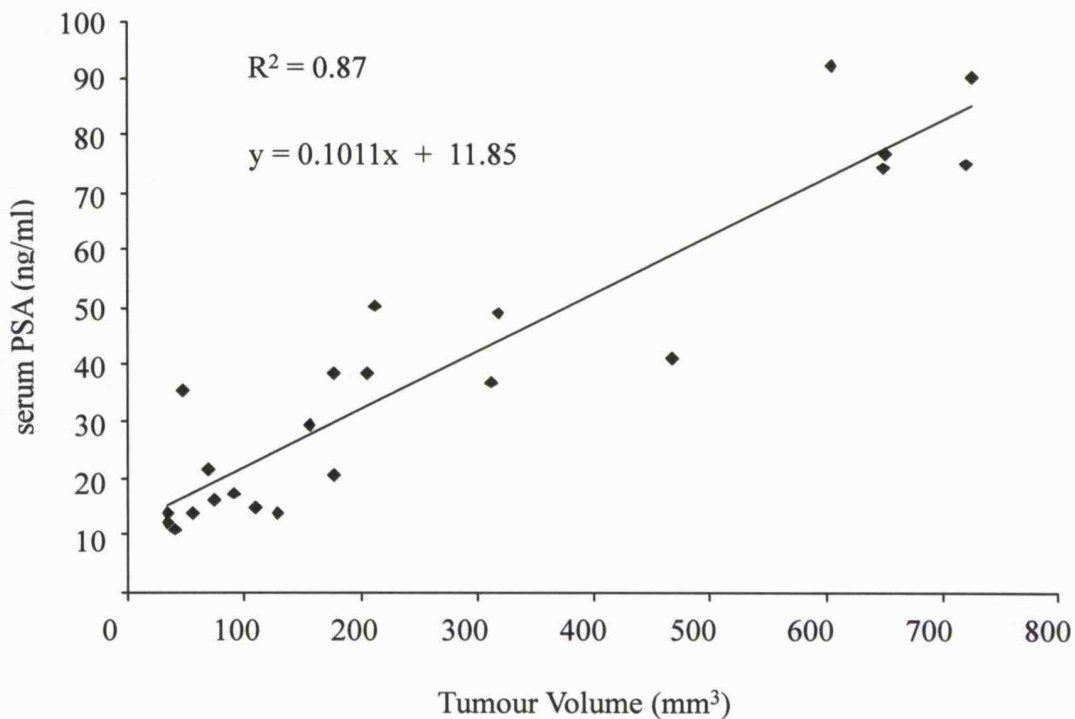


Figure 4.2.1.1. Effect of LNCaP tumour volume on serum PSA levels in the murine LNCaP xenograft. LNCaP human prostate cancer cells were implanted into the right flank of athymic nude mice (n=24). Tumour sizes were measured weekly with callipers. When tumours reached approximately 5 – 7 mm in length, mice were randomised into the experimental groups. All mice had their pre-treatment tumour volumes calculated using the formula; $\text{volume (mm}^3\text{)} = \pi / 6 (L_1 \times L_2 \times L_2)$, where L_1 and L_2 represented the longest and shortest lengths, respectively. The tail veins of the mice were cut and 100 μl of blood was used to determine the pre-treatment serum PSA levels.

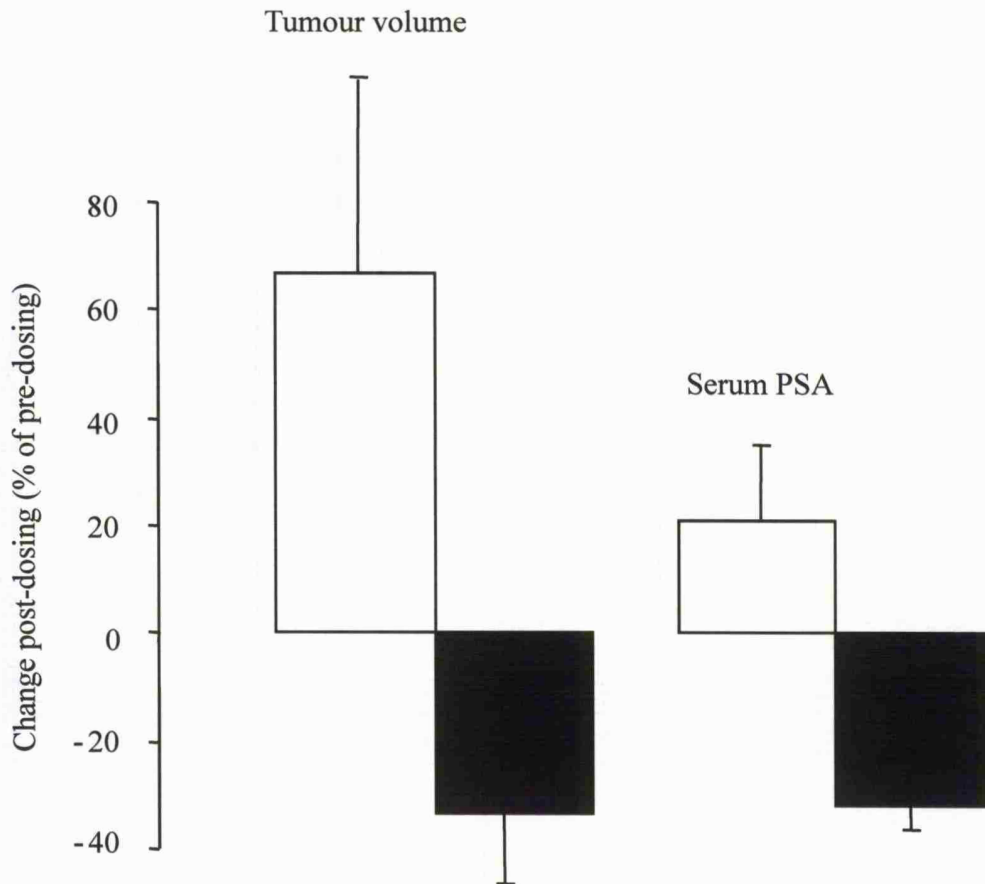


Figure 4.2.2.1. Effect of flutamide on tumour volume and serum PSA levels in the murine LNCaP xenograft. Open bars: control mice. Closed bars: treated mice. Control and treated mice received daily intra-gavage of water and flutamide (50mg/kg), respectively, for 7 days. Results are represented as percentage change from pre-dosing volume/levels. In control and treated mice, mean terminal tumour volumes and serum PSA levels were 506 ± 467 and $213 \pm 271 \text{mm}^3$, respectively, and 57 ± 31 and $21 \pm 14 \text{ ng / ml}$, respectively, (mean \pm SD, $n = 12$). When original pre- and post-dose values were compared, the difference was statistically significant ($p < 0.001$ by ANOVA) for both types of measurements. Following treatment, mice were killed by cardiac exsanguination. All pre- and post-treatment tumour volumes were calculated using the formula; volume (mm^3) = $\pi / 6 (L_1 \times L_2 \times L_2)$, where L_1 and L_2 represented the longest and shortest lengths, respectively. The pre- and post-treatment blood was used to determine PSA levels.

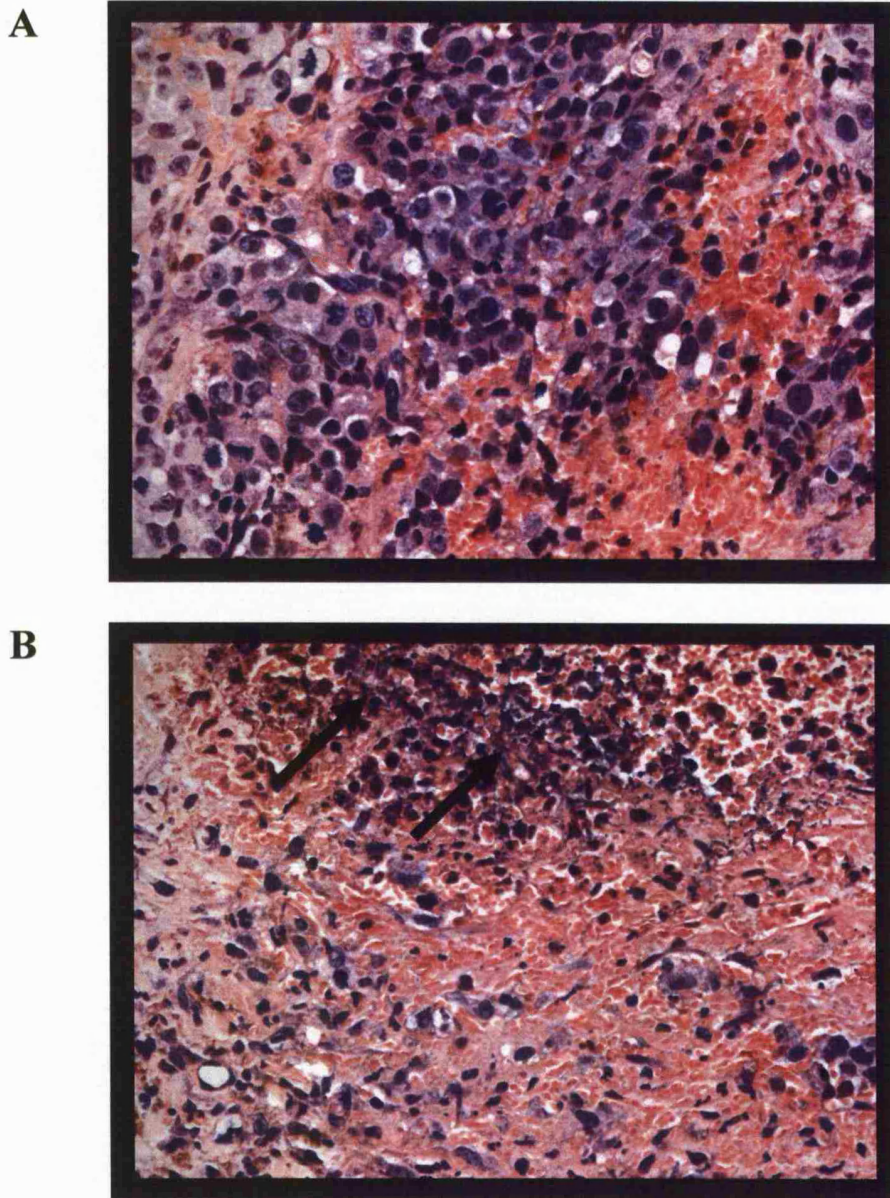


Figure 4.2.3.1. Effect of flutamide on the histological appearances of LNCaP tumours in the murine LNCaP xenograft. Representative photomicrographs of LNCaP tumours from a control mouse (A) that received vehicle-control and a mouse that received flutamide (50 mg / kg *ig*) daily for 7 days (B). Haematoxylin and eosin stain (H & E), magnification x 250. Areas of focal necrosis and cell debris are demonstrated by the arrows in (B), that were not present in (A). LNCaP tumours (n=4) were excised, fixed in formalin and embedded in paraffin wax. Sections of 5 μ m thickness were taken and H & E staining was performed (Courtesy of Dr Peter Greaves)

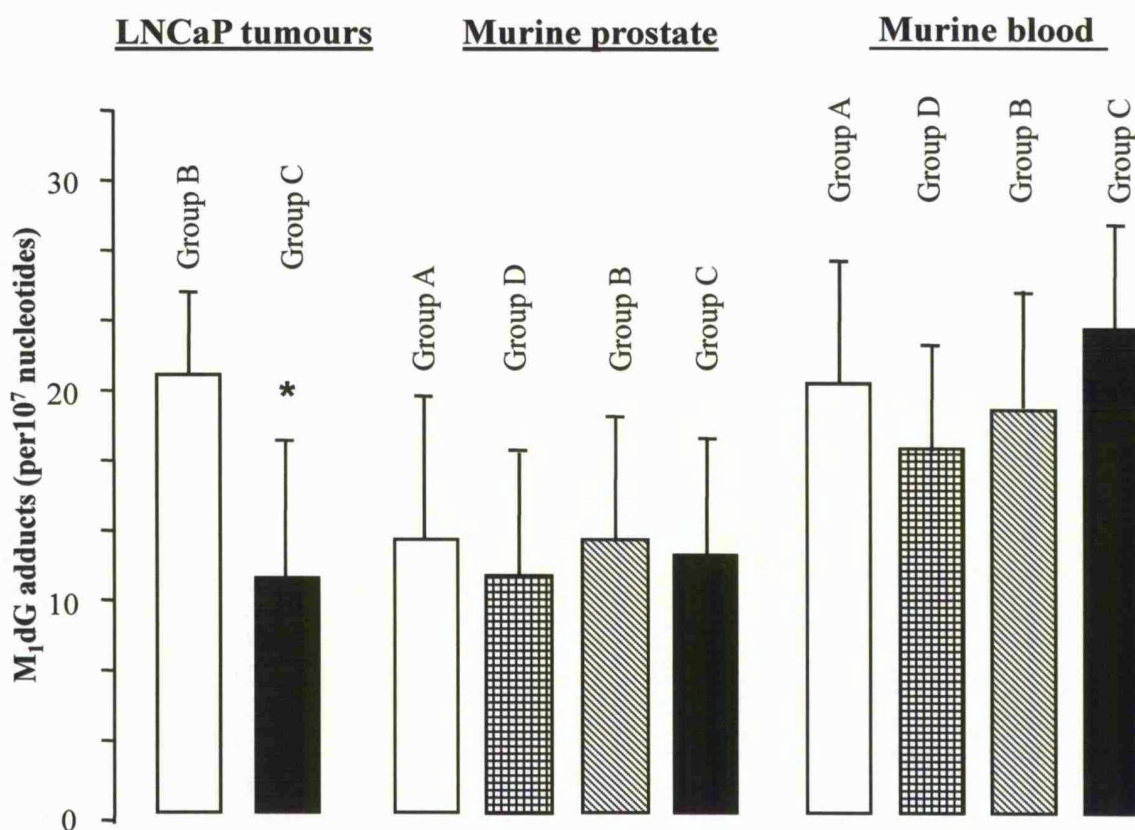


Figure 4.2.4.1 Effect of flutamide on M₁dG levels in LNCaP tumours, murine prostate glands and murine blood leucocytes. Group A represent mice not injected with LNCaP cells and acted as non tumour-bearing controls (n=5). Group B represent LNCaP tumour-bearing control mice (n=12). Group C represent LNCaP tumour-bearing mice treated with flutamide (n=12). Group D represent mice injected with LNCaP/Matrigel but did not develop tumours and acted as matrigel controls (n=9). Mice in Group B were treated daily with flutamide (50 mg/kg ig) for 7 days. Mice in Groups A, C and D were treated daily with vehicle-control for 7 days. Results are mean \pm SD. (*) indicates the difference to control mice is significant ($p < 0.001$, Mann-Whitney test). LNCaP tumours were implanted into the right flank of athymic nude mice. Tumour sizes were measured weekly with callipers, when tumour reached approximately 5 – 7 mm in length, mice were randomised into control and treatment groups. Following treatment mice were killed by cardiac exsanguination. The blood was used to determine M₁dG levels. LNCaP tumours and murine prostate tissues were excised, M₁dG levels were determined using the Qiagen and immunoslot blot methods.

4.3 Discussion

Of the 34 mice injected with LNCaP tumour cells, 25 mice developed palpable and measurable tumours (73.5%). Development of LNCaP tumour was approximately 60% and 100% in athymic nude and SCID mice, respectively, reported by Sato *et al.* (1997). Athymic nude mice are deficient only in matured T-cell immunity. In contrast, SCID mice are deficient in both T- and B-lymphocytes, which may account for their higher rates of LNCaP tumour development.

In athymic nude mice bearing the subcutaneous LNCaP tumours, increased tumour size correlated with increased serum PSA production (Figure 4.2.1.1). This positive correlation had previously been reported by Gleave *et al.* (1992).

LNCaP tumour growth and serum PSA levels were found to be significantly decreased in mice treated with flutamide compared with mice of the control group who received vehicle-control (Figure 4.2.2.1). This finding reflects the androgen antagonist properties of flutamide resulting in the inhibition of prostate cancer cell growth. These *in vivo* results are consistent with the effects of flutamide observed in LNCaP cells *in vitro*. Macroscopically, when LNCaP tumours of mice treated with flutamide and vehicle-control were assessed, areas of obvious necrosis were seen in the former which were absent in the latter (Figure 2.4.1.2A). Furthermore, histological examination of these tumours confirmed focal areas of necrosis of mice treated with flutamide which were absent in the controls (Figure 4.2.3.1). The findings of both a reduction in tumour volume and necrosis are features of anti-androgen therapy seen with flutamide (Raghow *et al.*, 2000).

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M₁dG levels were decreased by 46% in LNCaP tumours of mice treated with flutamide compared to vehicle-control ($p < 0.001$ by Mann-Whitney test). It has been shown that M₁dG levels may be affected by specific location in sampling of the tumour (Garcea *et al*, 2005). In this study, pre- and post-treatment levels of M₁dG were measured. Patients suffering from colorectal cancer received curcumin daily for 7 days; the control group did not receive curcumin prior to undergoing surgery for curative intent. Biopsies were taken pre-operatively from tumour edge, post-operatively from tumour edge and tumour core. M₁dG levels in the pre-operative tissue were 4-fold higher than those obtained from post-operative core tissue. Furthermore, a 2-fold difference in M₁dG levels was seen between post-operative tumour edges compared to tumour core tissue. This discrepancy may reflect that biopsy of tumour edge had predominantly tumour cells compared to tumour core. The relatively low levels of M₁dG in the latter may be a result of necrotic and/or stromal cells (Everett *et al*, 2001). The M₁dG levels taken from the LNCaP xenograft were unlikely to be due to this effect, given that considerable care was taken when sampling the tissue i.e. deliberately avoiding necrotic areas of the tumour.

It is noteworthy; that although a significant change in M₁dG levels were demonstrated in the LNCaP tumours *in vivo*, M₁dG levels in murine prostate tissues were not affected by flutamide (Figure 4.2.4.1). This discrepancy may relate to differences between normal and malignant prostate tissue in cellular responses to oxidative DNA damage. A study of human prostate tissue from patients with benign prostatic hypertrophy and prostate cancer offers further insight into the link between carcinogenesis and oxidative DNA damage

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(Giovannini *et al*, 2004). In this study, levels of repair proteins and 8-oxo-dG adducts were analyzed by Western blot and immunochemistry. The results suggested that oxidative DNA damage may drive neoplastic cells to activate repair mechanisms which favour escape from senescence and consequential clonal expansion of DNA-damaged clones, whereas increased oxidative DNA damage in non-malignant prostate cells may result in senescence. Analysis of human precancerous lesions indicates that DNA damage response and senescence markers co-segregate (Bartkova *et al*, 2006), suggesting that senescence and apoptosis in human preneoplastic lesions provide barriers to malignant progression and that malignant cells have acquired methods to overcome such barriers.

The role of androgen-induced oxidative stress in animal models has been explored by Siddiqui *et al*. (2005). Testosterone (subcutaneous injections of daily testosterone for 5 days) induced oxidative stress in Wistar rats. Prior to androgen administration, rats were fed aqueous tea extracts (ATE) at concentrations of 0.5, 1.0 and 1.5% for 15 days, the control groups received water. At the end of the experiments, prostate tissues were dissected out and the anti-oxidant enzymes, catalases, superoxide dismutases and glutathione-S-transferases were measured. The results showed that testosterone increased lipid peroxidation (LPO). Furthermore, LPO was decreased by pre-treatment of rats with ATE. LPO activity in the 0.5, 1.0 and 1.5% ATE groups was reduced by 86, 91 and 98%, respectively. Levels of anti-oxidant enzymes were also significantly decreased in the treatment groups. This study infers that androgens increase ROS as reflected by the levels of anti-oxidant enzymes and LPO activity. This effect may be

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prevented by the administration of ATE from black tea, known to contain strong anti-oxidant properties (Ho *et al.*, 1992). LPO is known to cause oxidative DNA damage with the resultant formation of M₁dG (Marnett, 1999). The results presented above are consistent with the notion that androgens increase oxidative stress and, therefore, M₁dG levels.

A potential link between androgen manipulation and levels of oxidative DNA damage in humans has been suggested by Miyake *et al.* (2004), who used a quantitative sandwich enzyme-linked immunosorbent assay to measure oxidative DNA adduct levels in urine samples from 82 patients with prostate cancer with correction for urinary creatinine levels, 40 of whom underwent hormonal manipulation. Although radical prostatectomy in 42 patients did not significantly affect levels of 8-oxo-dG in the pre- and post-surgical urine samples, in patients who received treatment with LHRH agonists and/or an anti-androgen, mean urinary 8-oxo-dG levels diminished by approximately 20% ($p < 0.05$ by Mann-Whitney test). These findings suggest that androgen suppression rather than prostate cancer regression may be directly involved in the decrease in oxidative DNA damage. Interestingly, although there was a significant decrease in the levels of the urinary oxidative DNA adducts, there was no change in levels of serum PSA. This finding suggests that oxidative stress may also be an early event in prostate carcinogenesis.

The clinical data from the study of Miyake *et al.* (2004) and the results from the xenograft model presented here are consistent with the hypothesis that the principal anti-oxidative effect of hormonal treatment is in malignant prostate

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tissue. Further support for this hypothesis is provided by the phase II trial performed in men undergoing radical prostatectomy for prostate cancer who received dietary supplements of tomato sauce containing high levels of the anti-oxidant, lycopene for 3 weeks prior to surgery (Chen *et al*, 2001). The investigators demonstrated that post-intervention levels of 8-oxo-dG, measured by HPLC with electrochemical detection, were significantly reduced in prostatic tissue compared to pre-intervention levels in the same patients. There was a statistically significant decrease in serum PSA levels from a mean pre-treatment level of 10.9 to a mean post-treatment level of 8.7 ng/ml. Clinically, over a longer timeframe such a decrease in serum PSA would not be considered significant, however, given that decrease was seen at 3 weeks of treatment, it would be interesting to know what effects a longer duration of treatment would have on serum PSA levels. This trial indicates that measurable levels of oxidative DNA adducts are present in human prostate tissue and such levels may be influenced by dietary agents over a short time frame. Given the reduction of oxidative DNA damage in prostate tissue was reflected in blood leucocytes, this also raises the possibility that 8-oxo-dG may serve as a surrogate biomarker in anti-oxidant chemoprevention trials of prostate cancer. In our study, we failed to observe this surrogate effect in blood leucocytes of mice. A plausible explanation for this difference may be that, unlike anti-androgens, lycopenes exert their anti-oxidant effects directly on blood leucocytes.

Since the detection of a metabolite of M₁dG by LC/MS has recently been reported in urine from healthy volunteers, corroborated by nuclear magnetic resonance spectroscopy and independent chemical synthesis (Otteneder *et al*, 2006), the

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detection of oxidative DNA adducts and their oxidative metabolites is currently regarded as a promising opportunity for the clinical development of biomarkers of endogenous DNA damage associated with inflammation, oxidative stress and carcinogenesis. It is important that highly specific and sensitive analytical methods are used for the analysis of urine samples, such as isotope-dilution LC-MS/MS (Hu *et al*, 2004).

CHAPTER FIVE

DISCUSSION

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Pre-clinical chemoprevention research aims to provide pharmacokinetic, pharmacodynamic and mechanistic data on compounds investigated as well as their efficacy and toxicity. It aims to explore the mechanisms of action of new agents primarily through *in vitro* studies in cells, followed by *in vivo* studies in models of human malignancy to assess efficacy. The discovery and development of chemopreventive agents is mechanistically driven, focusing on modulating molecular targets associated with biomarkers of carcinogenesis.

The ultimate aim of this project was to provide information that might help identify new biomarkers that may be used in trials of chemoprevention of prostate cancer. Oxidative DNA adducts, M₁dG and 8-oxo-dG were assessed as potential biomarkers. DNA adducts could be considered as important biomarkers for the exposure to genotoxic carcinogens, as they give an indication of the biologically effective dose of the carcinogen that has reached the tissue DNA under study (Farmer, 2004). A suitable biomarker of efficacy is one that is undetectable in normal tissue but is detectable during cancer development, such that the change in biomarker is related to carcinogenic progression and can be detected in target tissue easily accessible for multiple biopsies (Sharma *et al*, 2004). Ideally, it should be possible to measure biomarkers on samples that are readily obtainable, generally, this means blood and/or urine (Collins, 2002).

The particularly long latency period of prostate carcinogenesis and the potential reversibility of pre-malignant lesions (e.g. HGPIN); provides an ideal window of opportunity for intervention. This allows chemoprevention trials for prostate cancer using diet-derived agents or drugs that effect hormonal manipulation an

attractive option. However, at present these chemoprevention trials for prostate cancer are large of long duration and extremely expensive. One of the reasons is that the principal endpoints used in Phase III trials is cancer incidence, as clearly reflected by the PCPT, REDUCE and SELECT trials (see section 1.2.2).

The results shown here provide the first reported description of oxidative DNA adduct levels in human malignant prostate cancer cells grown *in vitro* and as a solid tumour in mice *in vivo*. The oxidative DNA adducts, M₁dG and 8-oxo-dG are promising potential biomarkers that may be used in prostate cancer chemoprevention. The suitability of these oxidative DNA adducts as a biomarker of pharmacological efficacy of potential chemopreventative agents has been investigated in several studies, particularly with anti-oxidative agents. Breast cancer prevention in the Tag mouse model was assessed with catechins and theoflavins, the principal anti-oxidant found in green and black tea, respectively, (Kaur *et al*, 2007). In this study, mice received the tea (0.05%) in drinking water for their lifetime. M₁dG levels in murine mammary tumours of treated mice were significantly reduced by 78 and 63%, respectively. In an earlier clinical study, median M₁dG levels in histologically normal breast tissue from patients with breast cancer were almost 3-fold higher than those from control subjects without breast cancer (Wang *et al*, 1996). M₁dG and 8-oxo-dG levels were significantly reduced by 39% and 24% in intestinal adenoma tissue of the Apc^{min+} mouse model treated with curcumin (0.2%) for 14 weeks (Tunstall *et al*, 2006). Curcumin is the major constituent of the yellow spice turmeric; it acts as a potent anti-oxidant (Jovanovic *et al*, 2001). Furthermore, M₁dG has been used as a

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potential biomarker to assess the efficacy of curcumin as a colorectal chemopreventive agent in a pilot clinical study (Garcea *et al.*, 2005).

Although the results are promising, there are issues that need to be addressed before M₁dG and 8-oxo-dG may be considered further as biomarkers for chemoprevention trials of prostate cancer. Firstly, common pathological features of prostate cancer are heterogeneity and multi-focality (Byar and Mostofi, 1972). The LNCaP cells used in both the *in vitro* and *in vivo* work are derivatives of a single clone of homogeneous malignant cells. Therefore, one needs to take this fact into consideration when extrapolating the use of such oxidative DNA adducts in clinical trials. Secondly, although a significant change in M₁dG levels was demonstrated in the LNCaP tumours *in vivo*, there was no difference observed in either the murine prostate glands or blood leucocytes. This discrepancy may relate to the differences between normal and malignant prostate tissue in cellular responses to oxidative DNA damage (Chapter 4). M₁dG levels did not significantly change in murine prostate tissue or blood leucocytes in flutamide-treated mice. This finding may be explained by the fact that oxidative stress in normal and malignant prostatic tissues is different. Finally, one of the limitations of the *in vivo* experiment was insufficient amount of DNA extracted from the blood of each mouse (approximately 20 – 30 µg). The quantity of DNA required for determining M₁dG and 8-oxo-dG levels is 3.5 µg and 50 µg, respectively. Therefore, we were unable to determine levels of 8-oxo-dG. The results of the *in vitro* work presented here suggest a correlation between both oxidative DNA adducts. One might speculate that a similar correlation would have been seen in the mouse model. Kadlubar *et al.* (1998) found a positive correlation between

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M₁dG and 8-oxo-dG levels in human pancreas tissue (Pearson's rank correlation test). Further work is needed to develop sensitive assays requiring less DNA than the current methods stipulate for the determination of 8-oxo-dG levels.

Men with prostate cancer deemed not suitable for curative therapy often undergo anti-androgen therapy by either medical or surgical castration. The principal aim of such therapy is depriving the prostate cancer cells of testosterone and thus, DHT. The biochemical effectiveness of such treatment is often measured in terms of a reduction of serum PSA levels. The development and validity of M₁dG and 8-oxo-dG as biomarkers should be assessed in a clinical trial. However, before assessing these biomarkers in a clinical trial, they should be evaluated in another pre-clinical model due to the limitations of the xenograft model. A feasible model would be to determine the effect of flutamide on M₁dG and 8-oxo-dG levels in the TRAMP mouse. Unlike, the nude mouse, the merits of using the TRAMP mouse would be the development of HGPIN and subsequently, the spontaneous development of prostate cancer of the murine prostate gland. However, the limitations of determining 8-oxo-dG levels in murine blood would still exist as experienced with the nude mouse, due to the small blood volume of the TRAMP mouse. Following this pre-clinical study, a clinical trial may be carried out, outlined in Figure 5.1.1. Men with histologically proven prostate cancer, who fulfil the inclusion criteria, undergo a pre-treatment and post-treatment (3 months) prostate biopsy to determine M₁dG and 8-oxo-dG levels. Prostate cancer is a multi-focal, heterogeneous cancer; therefore, additional prostate biopsies taken in these patients would provide the global effect of androgen deprivation on oxidative stress within the prostate gland of men with advanced prostate cancer,

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as reflected by M₁dG and 8-oxo-dG levels. Serum PSA levels taken at the same time points may reveal a corresponding correlation between M₁dG and 8-oxo-dG found in prostate tissues. Although, there was no correlation found between serum PSA and M₁dG levels in blood leucocytes and murine prostate tissue in our *in vivo* experiment, the murine prostate glands were considered normal with no evidence of malignancy. Therefore, the potential to develop such adducts as surrogate biomarkers in blood, still needs to be evaluated by determination of M₁dG and 8-oxo-dG in human leucocytes (additional 15-20 mls of blood required at each time point). Factors which effect oxidative stress and, therefore, oxidative DNA adducts need to be accounted for in the design of such a trial. Such factors include smoking and NSAIDS (Everett *et al*, 2001; Phillips, 2002); the latter may include patients who are on daily aspirin.

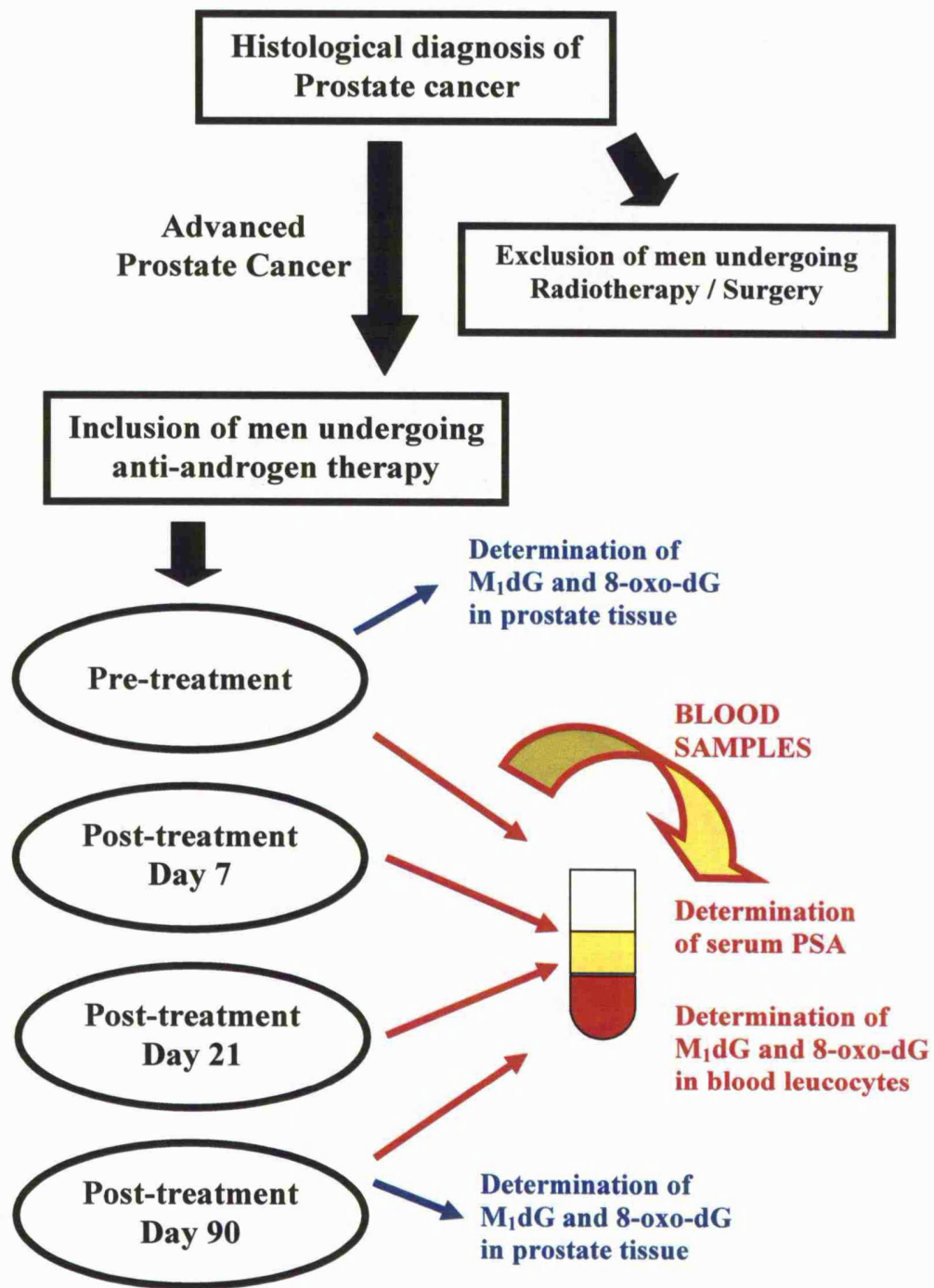


Figure 5.1.1 Design of an anti-androgen prostate cancer trial. The flow chart represents the design of a clinical trial to validate M₁dG and 8-oxo-dG oxidative DNA adducts as surrogate biomarkers in the treatment of advanced prostate cancer.

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An interesting and exciting secondary finding of our work suggests that oxidative DNA adducts may serve as biomarkers in chemoprevention studies using the anti-androgen, flutamide. HGPIN is potentially a reversible pre-malignant lesion of prostate cancer. However, one main drawback of using PSA levels to monitor the chemoprevention of prostate cancer directed at HGPIN is that levels do not change in HGPIN. Therefore, it is just conceivable that oxidative DNA adducts may serve as biomarkers to assess the efficacy of flutamide in this early stage of prostate carcinogenesis. The reluctance of using flutamide as a chemopreventive agent has been due to thrice daily preparation (750mg) and its associated side effects. However, Luo *et al.* (1997) showed that flutamide is just as effective at a reduced single daily dose of 250mg. The results of a recent 5 year prostate cancer chemoprevention study revealed that once daily dosing of flutamide was well tolerated. 172 men with HGPIN were randomised to receive once daily flutamide or placebo for 12 months. At 5 years, all men underwent prostate biopsies. The incidence of prostate cancer in placebo and flutamide treated men was 30.2% and 11.6%, respectively (Zhigang and Wenlu, 2008).

The results of the *in vitro* and *in vivo* work discussed here provide a mechanistic link between ROS and androgens. This is reflected by oxidative DNA damage measured by M₁dG and 8-oxo-dG levels in advanced prostate cancer cells. Currently, although the work has focused on advanced prostate cancer cells, we anticipate that future work will target pre-malignant and early prostate cancer. M₁dG and 8-oxo-dG may be used as potential biomarkers to assess the efficacy of new chemopreventive agents used in pre-clinical and clinical trials.

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Men that may fulfil entry criteria for such trials include;

- Men with an elevated serum PSA but with negative prostate biopsies.
- Men with early, low volume prostate cancer scheduled for active monitoring.
- Men with histological evidence of pre-malignant lesions e.g. HGPIN.

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APPENDICES

APPENDIX 1

Presentations

Pathak S, Sharma RA, Singh R, Farmer PB, Steward WP, Gescher AJ, Mellon K.

Chemoprevention of androgen-induced oxidative DNA damage by flutamide in human prostate cancer cells (poster).

British Association of Urological Surgeons

Harrogate, England

June 2004

Pathak S, Sharma RA, Singh R, Farmer PB, Steward WP, Gescher AJ, Mellon K.

Flutamide ameliorates oxidative DNA damage caused by DHT in human prostate cancer cells (poster).

95th Annual meeting of the American Association of Cancer Research

Florida, USA

March 2004

Pathak S, Sharma RA, Singh R, Farmer PB, Steward WP, Gescher AJ, Mellon K.

Flutamide ameliorates oxidative DNA damage caused by DHT in human prostate cancer cells. (poster)

Urological Research Meeting

London, England

Jan 2004

Sharma RA, Singh R, Garcea G, **Pathak S**, Steward WP, Gescher AJ, Farmer PB.

Do Oxidative DNA Adducts have a role in Clinical Chemoprevention? (oral)

2nd Chemoprevention meeting of the American Association of Cancer Research

Arizona, USA

Oct 2003

Pathak S, Sharma RA, Singh R, Farmer PB, Steward WP, Gescher AJ, Mellon K.

Androgens increase oxidative DNA adduct levels in human prostate cancer cells

(poster).

British Cancer Research Meeting

Bournemouth, England

July 2003

Pathak S, Sharma RA, Steward WP, Gescher AJ, Mellon K.

Effect of DHT on oxidative DNA adduct levels in human prostate cancer cells

(poster).

British Prostate Group

Edinburgh, Scotland

April 2003

Pathak S, Sharma RA, Steward WP, Gescher AJ, Mellon K.

Oxidative damage and Prostate Cancer. (oral)

Urological Club of Great Britain and Ireland

Leicester, England

Sept 2002

APPENDIX 2

Published abstracts

Pathak S, Sharma RA, Mellon K *et al.* Chemoprevention of androgen-induced oxidative DNA damage by flutamide in human prostate cancer cells. *BJUI* 2004; 93(4): 34 (abstract).

Pathak S, Sharma RA, Mellon K *et al.* Flutamide ameliorates oxidative DNA damage caused by DHT in human prostate cancer cells. *AACR* 2004;93(4): 570 (abstract).

Sharma RA, Singh R, Garcea G, **Pathak S** *et al.* Do Oxidative DNA Adducts have a role in Clinical Chemoprevention? *Frontiers in Cancer Prevention*: p123, 2004 (abstract)

Pathak S, Sharma RA, Mellon JK *et al.* Androgens increase oxidative DNA adduct levels in human prostate cancer cells. *BJC* 2003; 88(1): S55-S72 (abstract).

APPENDIX 3

Published papers

Pathak S, Singh R, Verschoyle V, Greaves P, Farmer PB, Steward WP, Mellon K, Gescher AJ, Sharma RA. Androgen manipulation alters oxidative DNA adduct levels in androgen-sensitive prostate cancer cells grown in vitro and in vivo. *Cancer Lett* 261 (1): 74-83, 2008

Pathak S, Sharma RA, Steward WP, Mellon K, Griffiths TRL, Gescher AJ. Oxidative Stress and cyclo-oxygenase activity in prostate carcinogenesis: Targets for chemopreventive strategies. *E J Cancer* 2005; 41: 61-70 (review).

Pathak S, Sharma RA, Mellon JK. Chemoprevention of prostate cancer by diet derived anti-oxidants and hormonal manipulation. *Int J Onc* 22: 5-13, 2003 (review).



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Cancer Letters 261 (2008) 74–83

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Androgen manipulation alters oxidative DNA adduct levels in androgen-sensitive prostate cancer cells grown *in vitro* and *in vivo*

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Received 20 August 2007; received in revised form 3 November 2007; accepted 5 November 2007

Abstract

Intracellular reactive oxygen species (ROS) may cause oxidative DNA damage, resulting in the formation of adducts such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) and the cyclic pyrimidopurine *N*-1, *N*² malondialdehyde-2'-deoxyguanosine (M₁dG). These adducts have been associated with carcinogenesis, genomic instability and clonal evolution. We tested two hypotheses in human prostate cancer cells grown *in vitro* and in a xenograft model: (1) treatment of androgen-sensitive cells with DHT increases levels of oxidative DNA adduct levels; (2) flutamide, a competitive androgen receptor antagonist, prevents DHT-induced changes. Levels of M₁dG and 8-oxo-dG adducts were determined by immunoslot blot and liquid chromatography–tandem mass spectrometry. M₁dG and 8-oxo-dG levels were significantly higher than control levels in LNCaP cells exposed to supra-physiological concentrations (25–100 nM) of DHT (both $P < 0.05$ by ANOVA). Flutamide pre-treatment completely prevented this increase. In the xenograft model, tumour levels of M₁dG were decreased by 46% ($P = 0.001$ by Mann–Whitney Test) in flutamide-treated animals compared to controls. The changes demonstrated suggest that oxidative DNA adducts may serve as biomarkers of the efficacy of androgen manipulation in chemoprevention trials.

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Keywords: Carcinogenesis; Testosterone; Flutamide; DNA damage

Abbreviations: AR, androgen receptor; DHT, dihydrotestosterone; MDA, malondialdehyde; ROS, reactive oxygen species; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; ig, intragastric; M₁dG, cyclic pyrimidopurine *N*-1, *N*² malondialdehyde-2'-deoxyguanosine; 8-oxo-dG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PSA, prostate specific antigen.

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1. Introduction

Cancer of the prostate is the most commonly diagnosed solid malignancy and the second commonest cause of cancer-related deaths in men living in developed countries [1]. In the United Kingdom, the incidence is likely to double over the next 20 years due to a combination of improved longevity of the general population and more widespread use of prostate specific antigen (PSA) testing [2]. It has become increasingly apparent that reactive oxygen species (ROS) may be an important aetiological factor in the development and progression of prostate cancer reviewed in [3]. Exogenous and endogenous ROS of biological significance include hydrogen peroxide, superoxide ions and hydroxyl radicals, which may attack lipids, proteins and DNA within cells [4]. Damage induced by ROS can modulate gene transcription and alter the expression of transcriptional factors implicated in carcinogenesis, including *c-fos* and *c-jun* oncogenes [5,6]. A “pro-oxidant state,” measured by an increase in ROS or a disruption of protective antioxidant systems, has been observed in prostate cancer and in the pre-malignant lesion, high grade prostatic intraepithelial neoplasia [7].

Levels of oxidative DNA adducts, such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) and the cyclic pyrimidopurine *N*-1, *N*'-2 malondialdehyde-2'-deoxyguanosine (*M*₁dG), reflect direct and indirect oxidation of DNA by ROS [8,9]. These adducts have been associated with mutagenesis and carcinogenesis in preclinical models (reviewed in [10]). Since “control” DNA from tissue unexposed to endogenous mutagens does not exist *in vivo*, correlative studies represent the only means of relating changes in DNA adduct levels to carcinogenic events or intervention with potentially chemopreventive agents.

Androgens play a key role in the development and progression of the majority of prostate cancers [11,12]. Indeed, prostate cancer rarely develops in men castrated before puberty [13]. In the androgen-sensitive human prostate cancer cell line, LNCaP, stimulation with androgens has been shown to result in increased levels of ROS [14]. The same androgen stimulation of androgen-insensitive DU145 cells did not influence levels of ROS [14]. In a more recent clinical study, patients with prostate cancer who underwent hormonal therapy demonstrated a significant decrease in mean urinary 8-oxo-dG levels after androgen deprivation therapy

compared to mean levels measured before therapy [15]. Collectively, these data suggest that oxidative stress is associated with prostate carcinogenesis and that androgen suppression may be linked to a decrease in oxidative DNA damage. Such damage may contribute to the development of the malignant phenotype in pre-malignant lesions or to genomic instability in established tumours which may feed the clonal evolution of the cancer [16,17].

In view of these results, we postulated that androgen stimulation increases levels of oxidative DNA adduct levels in androgen-sensitive prostate cancer cells and that flutamide, a competitive androgen receptor antagonist, prevents androgen-induced oxidative DNA damage. We tested these hypotheses in two preclinical model systems: LNCaP cells grown *in vitro* and the same cell line grown as a xenograft in athymic male mice.

2. Materials and methods

2.1. Cells and incubations

Human-derived malignant prostate cell lines, LNCaP, DU145 and PC3 were obtained from the European Collection of Cell Cultures (Salisbury, UK) and cultured in large flasks in RPMI 1640 (25 mM HEPES) with 1% sodium pyruvate (Sigma-Aldrich, Poole, UK) and 1% glutamax (Gibco, Paisley, UK) containing 10% foetal calf serum. Cells of less than 20 passages were seeded either in flasks (10^6) for oxidative DNA adduct measurements, or in 12-well plates (10^4 per well) for measurements of proliferation. Since foetal calf serum contains physiological levels of androgens [18], experiments with flutamide required cells to be serum-deprived for 24 h prior to daily treatment with dihydrotestosterone (DHT) and/or the competitive androgen receptor antagonist, flutamide. DHT, flutamide and ethanol (the vehicle for the former 2 agents) were obtained from Sigma-Aldrich (Poole, UK). Cells were treated with physiological (2.5 nM) or supra-physiological (5–100 nM) concentrations of DHT daily. Flutamide was added 30 min prior to DHT, to give a final concentration in medium of 5 or 10 μ M. The DHT and flutamide concentrations employed in these experiments were based on those shown to have pro-oxidant and anti-oxidant activity in LNCaP and DU145 cells in previous studies [14,19,20]. Control cells were exposed to the equivalent volume of ethanol. Cells were harvested using trypsin (1%).

2.2. Animals and treatments

Experiments were carried out under animal project licence PPL 40/2496 granted to the University of Leicester by the UK Home Office. The experimental design was vet-

ted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards set by the revised guidelines from the UK Coordinating Committee on Cancer Research (UKCCCR, London, UK). Male MF-1 outbred nude mice (30–40 g), obtained from Harlan UK (Bicester, Oxon, UK), were ear-punched for identification and maintained at 20–23 °C under conditions of 40–60% relative humidity and a 12-h light/dark cycle. Mice received an irradiated RM3 high protein rodent diet *ad libitum* and sterilised drinking water. LNCaP tumour cells were implanted and grown in nude mice as described previously [21]. After 7 days of acclimatization, animals were subjected to light anaesthesia with halothane (Concord Pharmaceuticals, Dunmow, UK), and LNCaP prostate tumour cells (5×10^6) suspended in 200 μ L of matrigel (Becton Dickinson, Worthing, UK) were injected subcutaneously into the right flank. Five mice were not injected as non-tumour controls. Mice were weighed weekly and assessed for tumour growth by measurement using callipers. Tumour volume was calculated by the formula: length \times width²/2, where length was the larger and width the smaller diameter of the tumour (in mm). When tumour size reached approximately 6–7 mm in diameter, mice were tail-bled to obtain blood (~100 μ L) for serum PSA measurement. At this time-point, mice (12 per group) were randomised to either treatment or control group. Mice received flutamide (50 mg/kg, formulated in 1.25% ethanol/water, dosage volume 3.33 mL/kg) *via* the *ig* route daily for 7 days. This dose was based on previous dose finding studies of flutamide in rodents [22,23]. Mice in the control group received the same volume of the vehicle *ig*. Mice that did not develop tumours were separated into control and treated groups ($n = 5$ each group) and dosed as described above. Twenty four hours after the seventh daily dose, tumour size was re-measured, mice were re-weighed and then exsanguinated under terminal halothane anaesthesia (cardiac puncture). Healthy prostate and tumour tissue were excised, weighed and frozen (–80 °C) prior to manual homogenization and DNA extraction (*vide infra*). Serum was obtained from blood by centrifugation, frozen and stored at –80 °C until analysis.

2.3. Histopathology

Some murine prostate tumours were fixed in formalin for a minimum of 2 weeks and then embedded in paraffin wax. Sections (5 μ m thick) were cut and stained with haematoxylin and eosin before microscopic examination.

2.4. Measurement of PSA, M₁dG and 8-oxo-dG DNA adduct levels

PSA in cellular supernatant or murine serum was measured by radioimmunoassay kit (3rd generation immulite assay, Diagnostic Products Corporation, CA, USA).

For oxidative DNA adduct measurement, genomic DNA was isolated from cells and murine tumour tissue by the Qiagen method (Qiagen, Crawley, UK) for analysis of M₁dG, or by the Wako DNA extractor WB kit (Wako Chemicals, Neuss, Germany) and sodium iodide (NaI) for the analysis of 8-oxo-dG. The extraction was performed according to the manufacturer's instructions. In the case of the "Wako" isolation method, additional incubations with RNase A (20 U), RNase T₁ (2 U) and proteinase K (100 μ g) were included prior to the addition of NaI (all obtained from Sigma-Aldrich). Levels of M₁dG adducts in cells or murine tissues were determined in triplicate by an immunoslot blot method, as described previously [24], using a murine monoclonal anti-M₁dG antibody (provided by Dr. L.J. Marnett, Vanderbilt University, TN, USA) and goat anti-mouse horseradish peroxidase conjugated secondary antibody (Dako, Ely, UK). The M₁dG adduct level in each sample was corrected for the amount of DNA bound to the filter as determined by propidium iodide staining [25]. The detection limit for M₁dG was approximately 0.5 adducts per 10⁷ nucleotides. Experiments using cells were normalised to number of viable cells used for DNA extraction.

Levels of 8-oxo-dG adducts in cells were determined by an immunoaffinity column purification liquid chromatography–tandem mass spectrometry (LC–MS/MS) method as described previously [26]. Prior to enzymatic digestion, a stable isotope [¹⁵N₅]8-oxo-dG internal standard was added to each DNA sample. Following enzymatic digestion, an aliquot (1 μ g) of the digested DNA was analysed by HPLC–UV to determine the level of 2'-deoxyguanosine. Each digested DNA sample was subjected to immunoaffinity column purification prior to analysis by LC–MS/MS. The LC–MS/MS consisted of a Waters Alliance 2695 separations module with a 100 μ L injection loop connected to a Micromass Quattro Ultima Pt. (Micromass, Waters Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. The temperature of the electrospray source was maintained at 110 °C and the desolvation temperature at 350 °C. Nitrogen gas was used as the desolvation gas (650 L/h) and the cone gas was set to zero. The capillary voltage was set at 3.20 kV. The cone and RF1 lens voltages were 42 V and 30 V, respectively. A HyPurity (Thermo Electron Corporation, Runcorn, UK), C₁₈, (1.0 \times 150 mm, 3 μ m) column connected to a Uniguard HyPurity C₁₈ (1.0 \times 10 mm, 3 μ m) guard cartridge and a KrudKatcher disposable pre-column (0.5 μ m) filter was eluted isocratically with 0.1% acetic acid/methanol (90:10, v/v) at a flow rate of 50 μ L/min. The collision gas was argon (indicated cell pressure 2.0×10^{-3} mbar) and the collision energy set at 12 eV. The dwell time was set to 200 ms and the resolution was one *m/z* unit at peak base. The samples were analysed in positive electrospray ionization MS/MS selected reaction monitoring mode (SRM) for the [M+H]⁺ ion to base [B+H₂]⁺ transi-

tions of 8-oxo-dG (*m/z* 284–168) and [$^{15}\text{N}_5$] 8-oxo-dG (*m/z* 289–173). The limit of detection of 8-oxo-dG was 2.0 fmol on the column. The level of 8-oxo-dG was determined in each sample from the ratio of the peak area of 8-oxo-dG to that of the internal standard [$^{15}\text{N}_5$]8-oxo-dG and normalised to the amount of 2'-deoxyguanosine observed for each sample following DNA digestion as determined by HPLC–UV.

2.5. Statistical analysis

Results were subjected to analyses of variance (ANOVA) and covariance with a 5% significance level using Minitab software (Minitab Inc., PA, USA). Plots of residuals were used to ensure that variances were homogeneous and that the residuals had a normal distribution. Non-parametric data were analysed by Mann–Whitney test.

3. Results

3.1. Oxidative DNA adduct levels in cells *in vitro* and effects of exposure to flutamide

Background levels of the oxidative DNA adducts, M_1dG and 8-oxo-dG were determined in LNCaP, DU145 and PC3 cells after incubation in medium containing 10% foetal calf serum (i.e. physiological levels of androgens – see above). Treatment of androgen-sensitive LNCaP cells with supra-physiological doses of DHT caused a significant increase in levels of both oxidative adducts (Fig. 1). In contrast, such an increase was not observed in the androgen-insensitive DU145 and PC3 cells. As DHT-treatment affected cell proliferation, oxidative DNA adduct levels were normalised with respect to

the number of viable cells used for DNA extraction: This correction did not alter the observed effect (data not shown). Under conditions of growth in the presence of serum, both types of oxidative DNA adduct were elevated in LNCaP cells over control levels at concentrations of DHT of 25 nM and above (Fig. 2).

To test the hypothesis that androgen receptor (AR) stimulation increases oxidative DNA adduct levels in androgen-sensitive cells, LNCaP cells were deprived of serum for 24 h and then incubated daily with a physiological concentration of DHT with or without pre-incubation (30 min) with the competitive AR antagonist, flutamide. Flutamide counteracted the increase in levels of M_1dG or 8-oxo-dG caused by DHT (Fig. 3). In fact, 10 μM flutamide completely abrogated the oxidative DNA adduct level increase caused by 100 nM DHT. In these experiments, it should be noted that flutamide affected LNCaP cell growth and generation of PSA after 24 h: flutamide (10 μM) reduced the growth of LNCaP cells grown in the presence of DHT (100 nM) by 70% and it reduced PSA in the supernatant of cells cultured in the presence of serum by 85%. Correction of adduct levels with respect to the number of viable cells used for DNA extraction did not alter the effect demonstrated (data not shown).

3.2. Effect of flutamide on LNCaP tumour growth, serum PSA and oxidative DNA adduct levels *in vivo*

To extend the results obtained in cells *in vitro* to a rodent model in which the same cancer cells could be grown under physiological conditions *in vivo*, experiments were conducted in athymic nude mice bearing a LNCaP xenograft. Seventy percent of the mice that received LNCaP cells subcutaneously developed measurable tumours. Compared to control mice, the presence of

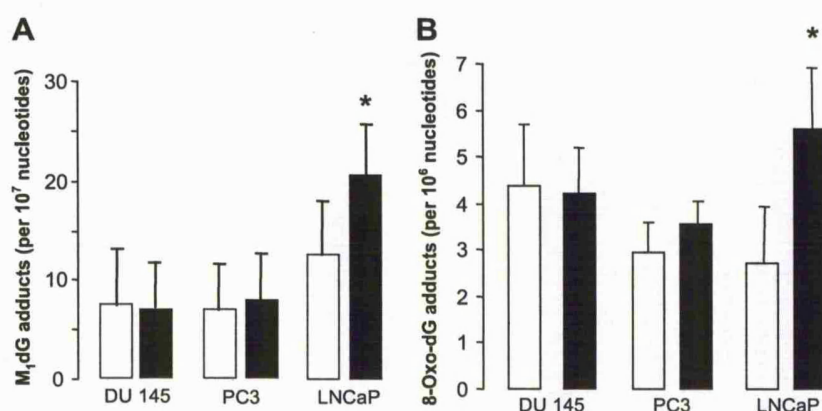


Fig. 1. Effect of daily treatment with DHT (100 nM) on levels of M_1dG (A) or 8-oxo-dG (B) in DU145, PC3 and LNCaP cells grown in medium. Open bars denote untreated cells and closed bars denote treated cells. Cells (approximately 10^6) were seeded in flasks and grown for 3 days in medium containing 10% foetal calf serum. DHT was then added daily for 7 days. Results (mean + SD) are presented from three separate experiments. Asterisks indicate that the difference is significant compared to control cells ($P < 0.05$ by ANOVA in A; $P < 0.01$ by Mann–Whitney test in B). For details of cell culture and measurement of M_1dG and 8-oxo-dG, see Section 2.

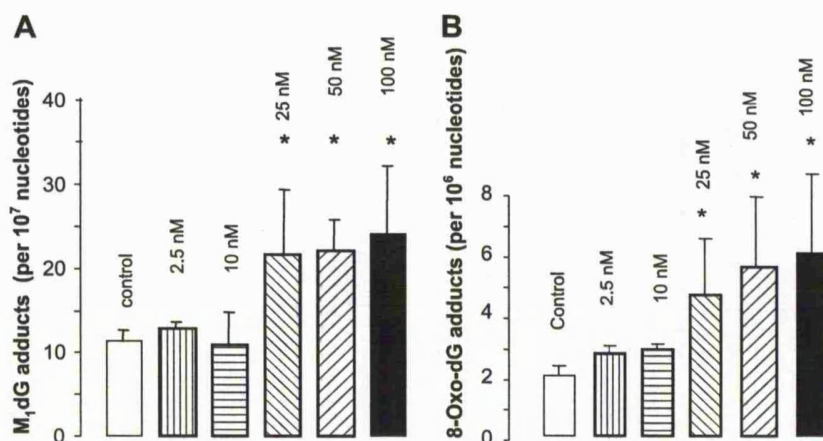


Fig. 2. Concentration dependence of effect of DHT on levels of M₁dG (A) or 8-oxo-dG (B) in LNCaP cells. Oxidative DNA adduct levels in incubations of cells omitting DHT are denoted "control" (open bars). Cells (approximately 10⁶) were seeded in flasks and grown for 3 days in medium containing 10% foetal calf serum. DHT was then added daily for 7 days. Results (mean + SD) are from three experiments. Stars indicate that the difference to respective control cells is significant ($P < 0.05$ by ANOVA). For details of cell culture and M₁dG or 8-oxo-dG measurements, see Section 2.

tumour did not affect murine bodyweight. Treatment with flutamide (50 mg/kg *ig* per day) for 7 days reduced mean tumour size and mean serum PSA concentration significantly, compared to animals which received vehicle only (Fig. 4). In the mice that did not develop measurable tumours, PSA was undetectable. When tumour tissue was subjected to pathological investigation, tissue from animals on flutamide displayed areas of focal necrosis, which were absent from mice which had received vehicle only (Fig. 5), consistent with an anti-neoplastic effect of flutamide.

M₁dG adduct levels were measured in tumour tissue, normal prostate tissue and peripheral blood cells. As shown in Fig. 6, administration of flutamide almost halved M₁dG levels in the tumour, whereas M₁dG levels in peripheral blood cells and benign prostate tissue were unaffected by flutamide treatment.

4. Discussion

The results shown here provide the first reported description of oxidative DNA adduct levels in human malignant prostate cells grown *in vitro* and as a solid tumour in mice *in vivo*. M₁dG formation can result from the reaction of malondialdehyde (MDA) with DNA, but other sources have been described [8–10]. In the absence of lipid peroxidation, oxidation of DNA by bleomycin *in vitro* gave rise to M₁dG adducts *via* base propenal formation [8]. Site-specific mutagenesis studies have suggested that mutation frequency caused by indirectly formed oxidative DNA adducts, such as

the M₁dG adduct, is similar to that of directly formed oxidative DNA adducts, such as 8-oxo-dG [27]. Moreover, clinical data have also indicated a relative insensitivity of M₁dG adduct levels to changes in MDA concentration in human tissues, when compared to the association of M₁dG adduct levels with 8-oxo-dG levels [9,28]. Based on these collective preclinical and clinical data, M₁dG adduct levels are currently considered biomarkers of oxidative DNA damage that reflect levels of ROS [29]. The correlative data for M₁dG and 8-oxo-dG adduct levels presented in the study described here provide further support for this hypothesis.

The LNCaP cells used in the study presented here were susceptible to the antagonist activity of flutamide, as reflected by cell growth inhibition and reduction in PSA production. Flutamide prevented increases in oxidative DNA adduct levels induced by supra-physiological concentrations of DHT. Although DHT levels vary within individuals (with circadian variation), between individuals and between tissues of one individual, a recent study of serum androgen levels in 132 men aged 30–72 years demonstrated a maximum titre of serum DHT around 2.0 nM with an inter-individual standard deviation of 0.15 nM [30]. These data are compatible with the findings of previous studies in which different assay techniques have been used and slightly higher means have been obtained [31,32]. Collectively, it can be stated that concentrations of

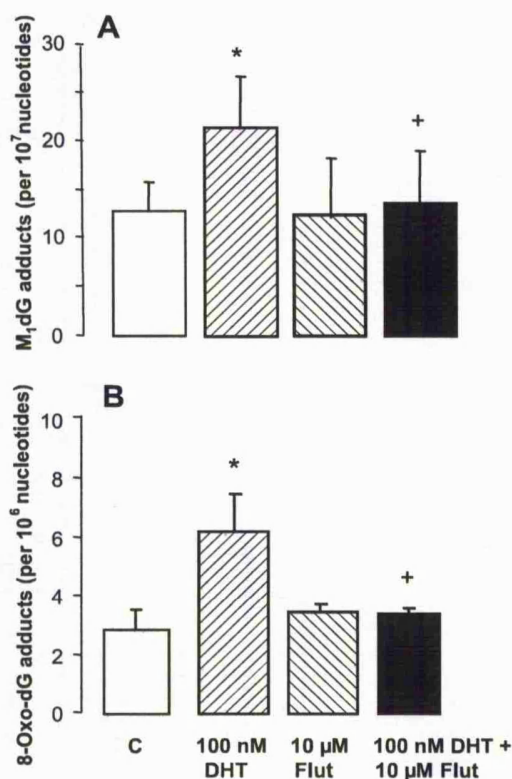


Fig. 3. Effect of flutamide (10 μ M) on increase in M₁dG (A) or 8-oxo-dG (B) caused by DHT (100 nM) in LNCaP cells. Oxidative DNA adduct levels in incubations of cells omitting DHT are denoted "control" ("C", open bars). Cells (10^6) were seeded in flasks and grown for 3 days in medium containing 10% foetal calf serum. Unlike the data shown in Figs. 1 and 2, cells were then grown in serum-free medium with or without the daily addition of flutamide and/or DHT for 4 days. Results (mean \pm SD) are from three experiments. Stars indicate that difference to control cells is significant, crosses indicate that the difference to cells incubated with DHT alone is significant ($P < 0.05$ by ANOVA). C, control; Flut, flutamide. For details of cell culture and M₁dG and 8-oxo-dG measurements, see Section 2.

DHT above 10 nM are regarded as above the physiological level observed in the serum of men. On account of the limitations of the cell culture model in reproducing low nanomolar concentrations of DHT in physiological circumstances, LNCaP cells were grown as xenografts on nude mice. In this model system, flutamide significantly reduced M₁dG adduct levels when administered at a dose which impeded tumour development and PSA generation, and the treatment caused tumour tissue necrosis (Fig. 5). The level of M₁dG adducts in the LNCaP tumours of untreated animals was similar to levels of adducts in cells grown *in vitro* stimulated with DHT.

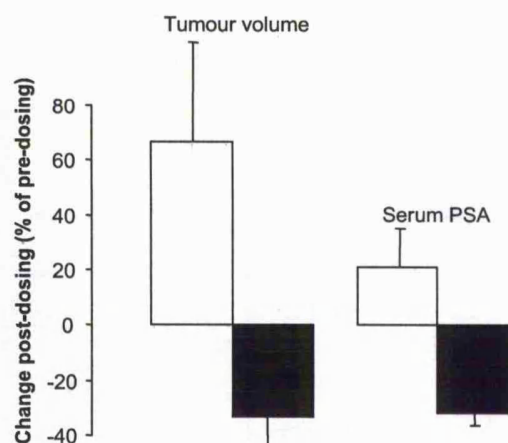


Fig. 4. Effect of treatment with flutamide (50 mg/kg *ig*) daily for 7 days on tumour volume and serum PSA in athymic MF1 mice bearing the LNCaP tumour. Open bars: control mice. Closed bars: treated mice. Results are presented as percentage change from pre-dosing volume/levels. In untreated animals, the mean terminal tumour volumes and PSA concentrations were 506 ± 476 mm³ and 57 ± 31 ng/mL (mean \pm SD, $n = 12$), respectively. When original pre- and post-dose values were compared, the difference was statistically significant ($P < 0.001$ by ANOVA) for both types of measurements. For details of tumour implantation and analyses of tumour volume and PSA, see Section 2.

The findings of the study presented here are compatible with the demonstration that stimulation of androgen-sensitive LNCaP cells with DHT results in increased levels of ROS [14]. Although it is not currently clear if androgen stimulation also affects detoxification enzymes for ROS in these cells, data from a xenograft model using androgen-responsive PC3 cells have suggested that androgens increase levels of ROS-detoxifying enzymes and conversely that androgen deprivation sensitises cells to the toxic effects of ROS [33]. These findings are consistent with the findings of an earlier study in male rats in which castration was associated with downregulation of certain ROS-detoxifying enzymes and an associated increase in the proportion of cells with high nuclear staining for 8-oxo-dG detected by immunohistochemistry [34]. Although definite conclusions cannot be drawn, it currently appears that the link between androgen stimulation and increased oxidative DNA adduct levels in androgen-sensitive cells is more likely to be attributable to increased generation of ROS rather than diminished detoxification. There are no data currently available on the potential effects of androgen stimulation on specific DNA repair enzymes, such as 8-oxoguanine DNA

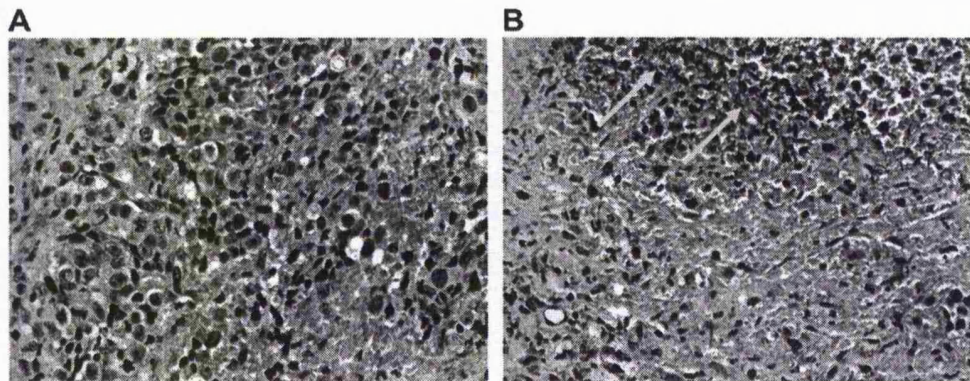


Fig. 5. Representative photomicrographs of LNCaP tumours from control mice (A) and mice that received flutamide (50 mg/kg *ig*) daily for 7 days (B). Haematoxylin and eosin stain, magnification 250 \times . Areas of focal necrosis and cell debris are demonstrated by arrows in B.

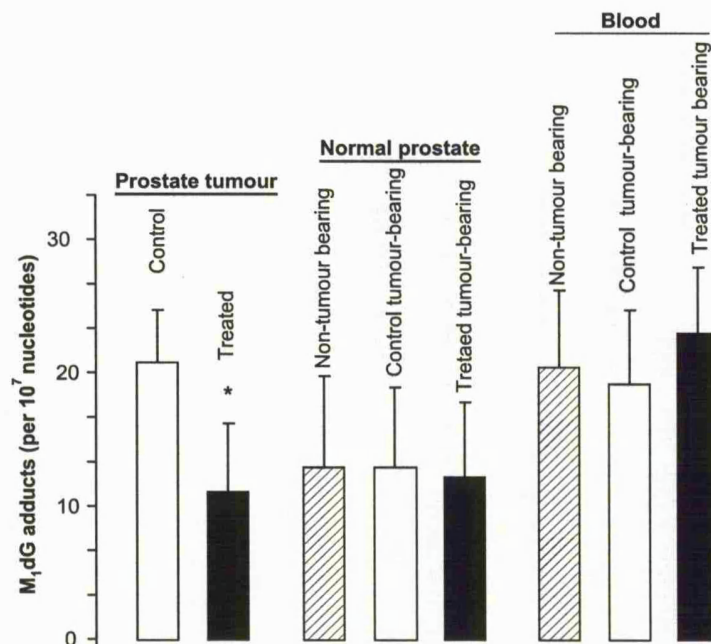


Fig. 6. Effect of treatment with flutamide (50 mg/kg *ig*) daily for 7 days on M₁dG levels in xenografted prostate tumour, normal prostate tissue and blood leukocytes from non-tumour-bearing mice or mice bearing LNCaP tumours. Open bars: control tumour-bearing mice (treated with vehicle only). Hatched bars: non-tumour-bearing mice (treated with vehicle only). Closed bars: tumour-bearing mice treated with flutamide. Results are mean \pm SD from 12 mice. Stars indicate that the difference to control mice is significant ($P < 0.001$ by Mann-Whitney test). For details of tumour implantation and measurement of M₁dG levels, see Section 2.

glycosylase (OGG1), involved in the repair of oxidative lesions.

It is noteworthy that, although a significant change in M₁dG levels was demonstrated in the LNCaP tumours *in vivo*, M₁dG levels in normal prostate tissue under the same conditions were not affected. This discrepancy may relate to differences between normal and malignant prostate tissue in

cellular responses to oxidative DNA damage. A recent study of human prostate tissue from patients with benign prostatic hypertrophy and prostate cancer offered further insight into the link between carcinogenesis and oxidative DNA damage [35]. In this study, levels of repair proteins and 8-oxo-dG adducts were analysed by Western blot and immunohistochemistry, respectively. The results suggested that

oxidative DNA damage may drive neoplastic cells to activate repair mechanisms which favour escape from senescence and consequential clonal expansion of DNA-damaged clones, whereas increased oxidative DNA damage in non-malignant prostate cells may result in senescence. Analysis of human precancerous lesions indicates that DNA damage response and senescence markers co-segregate [36], suggesting that senescence and apoptosis in human preneoplastic lesions provide barriers to malignant progression and that malignant cells have acquired methods to overcome such barriers.

A potential link between androgen manipulation and levels of oxidative DNA damage has been suggested by Miyake et al. [15], who used a quantitative enzyme-linked immunosorbent assay to measure oxidative DNA adduct levels in urine samples from 82 patients with prostate cancer (with correction for urinary creatinine levels), 40 of whom underwent hormonal manipulation. Although radical prostatectomy in 42 patients did not significantly affect levels of 8-oxo-dG in the pre- and post-surgical urine samples, in patients who received treatment with luteinizing hormone-releasing hormone analogue injections and/or an anti-androgen, mean urinary 8-oxo-dG levels diminished by approximately 20% ($P < 0.05$ by Mann–Whitney test). Since the detection of a metabolite of M₁dG by LC/MS has recently been reported in urine from healthy volunteers, corroborated by nuclear magnetic resonance spectroscopy and independent chemical synthesis [37], the detection of oxidative DNA adducts and their oxidative metabolites is currently regarded as a promising opportunity for the clinical development of biomarkers of endogenous DNA damage associated with inflammation, oxidative stress and carcinogenesis. It is important that highly specific and sensitive analytical methods are used for the analysis of urine samples, such as isotope-dilution LC–MS/MS [38].

The clinical data from the study of Miyake et al. [15] and the results from the xenograft model presented in the study reported here are consistent with the hypothesis that the principal anti-oxidative effect of hormonal treatment is in malignant prostate tissue. Further support for this hypothesis is provided by a phase II trial performed in men undergoing radical prostatectomy for prostate cancer who received dietary intervention in the form of supplements of tomato sauce containing high levels of the anti-oxidant, lycopene for 3 weeks prior to surgery [39]. The investigators demonstrated that

post-intervention levels of 8-oxo-dG, measured by HPLC with electrochemical detection, were significantly reduced in prostatic tissue compared to pre-intervention levels in the same patients. This trial indicates that measurable levels of oxidative DNA adducts are present in human prostate tissue and that levels may be influenced by dietary supplementation over a short time frame.

The results presented in the study reported here extend these findings to suggest that oxidative DNA adducts in malignant prostate tissue may reflect a mechanistic link between androgen sensitivity and DNA damage. If future studies corroborate such an association in humans *in vivo*, oxidative DNA adduct levels could be developed as potential biomarkers of the efficacy of androgen manipulation in the chemoprevention studies of androgen manipulation which are currently following the precedent set by the Prostate Cancer Prevention Trial [40].

Acknowledgements

This study was funded by grants from the UK Prostate Research Campaign (to R.A.S.) and Cancer Research UK (to R.A.S.) and the UK Medical Research Council (to A.J.G.). We thank Dr. L.J. Marnett (Vanderbilt University, TN, USA) for provision of primary antibody against M₁dG, S. Euden (University of Leicester, UK) and J. Edwards (MRC Toxicology Unit, University of Leicester) for technical support and Dr. H. McCarthy (Queen's University, Belfast, UK) for advice.

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Review

Oxidative stress and cyclooxygenase activity in prostate carcinogenesis: targets for chemopreventive strategies

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Received 16 October 2003; received in revised form 8 September 2004; accepted 30 September 2004
Available online 6 November 2004

Abstract

Over the last decade, epidemiological, experimental and clinical studies have implicated oxidative stress in the development and progression of prostate cancer. Oxidative stress may be linked to the effects of androgens, anti-oxidant systems and the pre-malignant condition, high-grade prostatic intraepithelial neoplasia. Cyclooxygenase-2 activity has been linked with prostate carcinogenesis. Evidence suggests that oxidative stress and cyclo-oxygenase-2 activity may be mechanistically linked. Agents such as anti-oxidants and cyclo-oxygenase-2 inhibitors may be of value in the chemoprevention of prostate cancer. The feasibility of intervention with such agents will depend on the development and validation of biomarkers for clinical trials, particularly markers of oxidative damage caused by reactive oxygen species (ROS). A greater understanding of the molecular events associated with oxidative stress will enhance the development of such biomarkers and should result in better strategies for the chemoprevention of prostate cancer. © 2004 Elsevier Ltd. All rights reserved.

Keywords: Chemoprevention; Prostaglandin; Androgens; DNA adducts

1. Introduction

Cancer of the prostate is the most commonly diagnosed solid malignancy and the second leading cause of cancer-related deaths in men living in developed countries [1]. Government statistics show that in the United Kingdom (UK) over 20000 new cases of prostate cancer are diagnosed and approximately 9000 men die as a result of the disease per annum [2]. The incidence is predicted to double over the next 20 years in the UK, mainly due to a combination of improved overall longevity of the general population and the more widespread use of prostate-specific antigen (PSA) testing, as

has already been noted in North America following prostate cancer screening [3,4]. Therefore there exists a strong need for a greater understanding of the molecular events in early prostate carcinogenesis and the potential interventional opportunities to prevent or slow the progression of the disease.

Over the last decade, it has become apparent that oxidative stress may be an important aetiological factor in the development and progression of prostate cancer. The impetus for experimental and clinical research into the role of oxidative stress and prostate cancer has increased following important secondary analyses of two clinical trials. The α -Tocopherol and β -Carotene study (ATBC study) was a double-blind, randomised, placebo-controlled trial of 29133 male smokers receiving α -tocopherol, β -carotene, ATBC in combination, or a placebo. Lung cancer incidence was the principal

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endpoint measured. Paradoxically, the trial showed an increased incidence of lung cancer amongst one subgroup of men given β -carotene supplements. However, secondary analysis of the data at six years follow-up demonstrated a 32% reduction in the incidence and a 41% reduction in mortality from prostate cancer among men who received supplementary α -tocopherol [5]. In a randomised controlled study of non-melanoma skin cancer prevention, 1312 patients were randomised to receive selenium supplements or placebo. The study did not show a protective effect on skin cancer incidence; however, secondary analysis at follow-up showed a 60% reduction in the incidence of prostate cancer [6]. Amongst the principal biological mechanisms of selenium and α -tocopherol (vitamin E) are anti-oxidant actions involving the quenching of reactive oxygen species (ROS) [7,8].

The concept that oxidants may play a role in the aetiology of prostate cancer or precursor lesions is relatively new. In this article, the evidence which links oxidative events with the disease process is reviewed. Furthermore, potential interventional strategies which exploit anti-oxidant activity in the prevention of prostate carcinogenesis are discussed.

2. Prostate carcinogenesis

Prostate cancer is rarely seen in men before the age of 50 years. On autopsy, the incidence of histological prostate cancer is 80% in men above 80 years of age, although the vast majority of these lesions are clinically insignificant. Prostate cancer is a global, multi-focal disease. Anatomically, greater than 70% of cancers occur in the peripheral zone of the prostate gland. The methods used in the diagnosis of prostate cancer include physical examination of the prostate (that might easily miss small or centrally placed tumours), serum PSA testing (that is not specific for prostate malignancy) and tissue biopsy (sampling error may miss malignancy). For example, prostate cancer has been detected in approximately 30–40% of men having repeat biopsies, having shown only high-grade prostatic intraepithelial neoplasia (HGPIN) on their initial biopsies [9]. The natural molecular history of prostate cancer suggests that HGPIN and, more recently, proliferative inflammatory atrophy (PIA) are pre-malignant lesions (see below).

3. Oxidative stress

Oxidative stress is defined as a disturbance in the equilibrium between ROS and detoxifying anti-oxidant systems; an excess of ROS leads to oxidative damage to cellular constituents (Fig. 1). The balance is normally

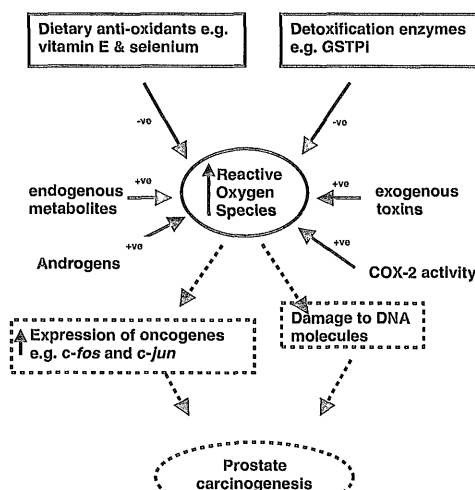


Fig. 1. Homeostasis of reactive oxygen species (ROS) metabolism: the potential for ROS to induce prostate carcinogenesis. Key: increase in ROS production (+ve), decrease in ROS production (-ve), malignant transformation pathway (→).

maintained by two principal mechanisms which render ROS less harmful. Firstly, intracellular anti-oxidants, such as vitamin C, vitamin E and selenium quench ROS. Secondly, anti-oxidant enzymes, such as glutathione *S*-transferase (GST)- π catalyse the conjugation of ROS to glutathione. Common ROS of biological significance include hydrogen peroxide, superoxide ions and hydroxyl anions. Sources of ROS can be subdivided into endogenous and exogenous, both types causing oxidative damage to important cellular molecules, including lipids, proteins and DNA [10].

ROS may react with DNA bases to produce oxidative DNA adducts. Such adducts have been associated with mutagenesis and carcinogenesis [11]. ROS may directly damage DNA leading to the formation of the DNA adduct, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG). Indirect actions of ROS include lipid peroxidation of cellular membranes, resulting in the production of compounds (e.g. malondialdehyde) which are capable of forming DNA adducts such as a pyrimidopurinone adduct of deoxyguanosine [11]. ROS can also increase the expression of transcriptional factors including *c-fos* and *c-jun* oncogenes involved in neoplastic transformation [12–14]. It has been shown that oxidative stress can be measured in prostate cancer cells [15,16], and that DNA damage can be strongly linked to oxidative damage [17]. A pro-oxidant state, which can be achieved by either an increase in ROS

or a disruption of the normally protective anti-oxidant systems, has been observed in both HGPIN and prostate cancer [18].

4. Proliferative inflammatory atrophy and high-grade prostatic intraepithelial neoplasia

The association of chronic inflammation with the development of human cancers is well recognised [19]. It is likely that ROS released by inflammatory cells during cycles of cellular damage and regeneration in these organs, result in permanent DNA damage [20].

The prostate gland is a common site for chronic inflammation. Although most focal prostatic atrophy lesions are considered quiescent, prostatic epithelial cell proliferation is increased in some lesions, thus focal prostatic atrophy, which is associated with chronic inflammation, is considered to be proliferative [21]. Recently, it has been suggested that PIA may develop into HGPIN and/or directly into prostate cancer. Unlike diffuse atrophy which is seen in androgen blockade therapy, PIA represents focal atrophy of certain regions of the prostate gland. Lesions previously reported as sclerotic atrophy and post-atrophic hyperplasia may be reclassified as PIA [22]. PIA is not routinely evident with standard haematoxylin and eosin staining, but can be recognised by special immunohistochemical staining, such as Ki-67 [23] and 34 β E12 [24], and may be recognised by groups of highly proliferative prostatic epithelial cells intermingled with inflammatory cells [23]. It has been hypothesised that PIA is a pre-malignant condition similar to pre-malignant inflammation observed in the liver and large bowel [21]. Oxidative stress may play a role in PIA and HGPIN [23].

HGPIN is a lesion that is now widely accepted as a pre-malignant condition for prostate cancer development [25,26]. It has been suggested that HGPIN may take 20–30 years to develop and that progression to clinically significant cancer may take between 3 and 15 years [27]. HGPIN may be a reversible condition, and the potential for reversibility has been shown by androgen deprivation therapy [28]. In this trial, men diagnosed with prostate cancer were randomised into two groups prior to their radical prostatectomies, a treatment group to receive androgen deprivation therapy for three months, and the control group received no therapy prior to surgery. Histological features showed a statistically significant decrease in the incidence of HGPIN and stage of prostate cancer in the treated group. Although this trial showed histological alterations in HGPIN lesions with anti-androgen therapy, there is no evidence in well controlled clinical trials that this equates to a decrease in clinical progression of the disease.

5. The role of cellular anti-oxidant enzymes

Cellular anti-oxidant enzymes include GSTs, dismutases, catalase, glutathione peroxidase and glutathione reductase. Most studies in prostate cancer have focused on the first three of these enzyme systems, especially the GST superfamily. The potential damage inflicted upon cellular proteins and DNA due to the generation of ROS may be prevented by a number of anti-oxidant enzyme systems detoxifying ROS. For example, GST π catalyses the conjugation of ROS to glutathione, thus rendering the reactive species less capable of reacting with cellular structures such as DNA (Fig. 1).

5.1. Glutathione S-transferases

The most extensively studied anti-oxidant enzyme in prostate cancer is the π class GST. In particular, expression of GSTP1 has been extensively studied in human prostatic tissues. Two thirds of normal basal prostatic epithelial cells immunostain intensely for GST π , whereas acinar epithelia stain weakly for GST π [29]. Immunopositivity for GST π is rare in HGPIN and incidental prostate cancer [30]. Interestingly, GST π expression is increased in PIA, possibly in response to oxidative stress [23]. It has been postulated that some clones within PIA lesions are incapable of expressing GST π , potentially as a result of a gene defect [28]. Lesions may then become repetitively damaged by ROS, subsequently developing into HGPIN or prostate cancer.

Even in the presence of a normal *GSTP1* gene, it is known that hypermethylation of the *GSTP1* gene promoter can result in failure to transcribe the gene [18]. Hypermethylation of *GSTP1* gene CpG island is present in a subset of PIA lesions, but not in normal or hyperplastic epithelium of the prostate [31]. Indeed, hypermethylation is seen in more than 70% of HGPIN lesions [32], and more than 90% of prostate cancer [33]. This epigenetic alteration may explain the vulnerability of HGPIN lesions and prostate cancer to oxidative cellular damage. In a detailed study using laser-capture microdissection, hypermethylation of the *GSTP1* gene was seen to be present in some PIA lesions. However, this was not present in normal or hyperplastic epithelia of the prostate [31], supporting the hypothesis that these subsets of PIA lesions may lead to HGPIN and/or prostate cancer development.

Other members of the GST superfamily have been less well studied in prostate carcinogenesis. Increases in the α class GST expression have been implicated in the pathogenesis of human cancers, including renal and bladder cancers [34]. Recently, in a study of radical prostatectomy specimens, high levels of GSTA1 immunoreactivity were reported in PIA lesions, whereas low levels were found in HGPIN and prostate cancer [35].

This finding further suggests a lack of detoxification activity in HGPIN and prostate cancer.

5.2. Dismutases

Dismutases catalyse the conversion of superoxide radicals to water. There are two major forms of the superoxide dismutase (SOD). Intracellular copper–zinc SOD1 is found in the nucleus and cytoplasm. Manganese superoxide dismutase (SOD2) is found in mitochondria. Studies have found SOD1 and SOD2 immunoreactivity to be lower in prostate cancer cells than in benign cells [36,37]. The selective immunoreactivity for SOD1 was found to be more nuclear than cytoplasmic in benign cells and cytoplasmic in prostate cancer cells [37]. A further study showed that nuclear immunoreactivity to SOD2 was greater in metastatic than primary prostate cancer cells [38]. Since ROS induce antioxidant enzyme systems, a plausible explanation could be that, in general, the growth rates of metastatic prostate cancer cells tend to be much greater than primary prostate cancer cells; and the greater oxidative stress seen in the former cells reflects progression of the disease.

In contrast to these immunohistochemical studies, enzyme activity assays for SOD1 and SOD2 have shown similar levels of activity in benign and malignant prostatic epithelial cell cultures [39]. However, enzyme activity was assessed in cellular homogenates that were contaminated with normal cells, which may make it difficult to draw definite conclusions from the enzyme activity study.

5.3. Catalase

Catalase (CAT) catalyses the conversion of hydrogen peroxide to water and is located within peroxisomes and the cytoplasm of cells. Two studies have shown lower CAT immunoreactivity in prostate cancer than in benign prostate tissue [36,37]. Lower CAT immunoreactivity has also been shown in HGPIN compared with benign prostatic tissue [37]. By contrast, another study detected increased CAT activity in prostate cancer tissue compared with benign prostate tissue [40]. Another enzyme assay has detected similar levels of CAT activity in prostate cancer tissues and primary human prostate cells [39]. Conflicting results are not uncommon in the measurement of short-lived and highly reactive indices, such as assays that aim to directly measure ROS. An indirect, but more stable, and perhaps more biologically relevant, indicator of oxidative stress is the measurement of the levels of the products of oxidative damage, such as protein or oxidative DNA adducts. Such markers have been found to be elevated in metastatic prostate cancer cells compared with primary cancer cells [38].

5.4. Potential interventions

A number of interventional strategies are under development in an attempt to rectify deficiencies in the levels of anti-oxidant enzymes during the early stages of prostate carcinogenesis. Supplementary anti-oxidants may be given to patients as capsules or as part of the dietary matrix [41]. Alternatively, it may be possible to augment the expression of other anti-oxidant or detoxification enzymes, thus compensating, at least partly, for low levels of activity of enzymes such as GST π . An agent which may be capable of favourable enzyme induction, oltipraz, is currently being developed in large-scale clinical trials in the chemoprevention of hepatocellular carcinoma in China [42], and its potential to prevent bladder cancer is also under scrutiny. Its effects on human prostate cells are not yet known.

6. The role of arachidonic acid metabolism

Diets rich in ω -3 polyunsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid) found in fish oils may reduce the risk of prostate cancer [43]. ω -6 Polyunsaturated fatty acids such as arachidonic acid and its precursor, linolenic acid, are major ingredients of vegetable oils: they are consumed in high quantities in the typical Western diet, and may be relevant to the pathogenesis of prostate cancer [44]. Oxidative lipid peroxidation accompanying the biosynthetic metabolism of arachidonic acid from membrane phospholipids to prostaglandins is a possible contributor to prostate carcinogenesis [45].

6.1. Cyclo-oxygenase enzymes

Cyclo-oxygenase is a rate-limiting enzyme in prostaglandin biosynthesis, a two-step enzymatic process in which ROS are generated as reviewed by Hussain et al. [46]. Firstly, arachidonic acid is converted to prostaglandin G₂ by the oxygenase activity of cyclo-oxygenase, prostaglandin G₂ then undergoes peroxidation to prostaglandin H₂ (Fig. 2). Unlike cyclo-oxygenase-1 (COX-1), COX-2 is normally undetectable in most tissues, but it is inducible by a variety of stimuli, including mitogens, growth factors and cytokines [47]. Recent studies have shown that overexpression of COX-2 is sufficient to induce breast tumours in transgenic mice, subsequent inhibition of the COX-2 pathway resulted in a reduction in tumour incidence and progression [48]. In actual prostate tissue specimens, COX-2 expression remains controversial. Earlier data suggested COX-2 expression was significantly higher in cancerous than in benign prostate tissues when assessed by immunohistochemistry [49–51], Western blotting [52] and reverse transcription-polymerase chain reaction (RT-PCR)

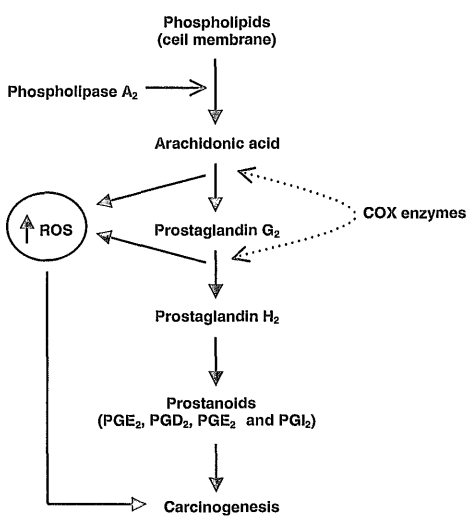


Fig. 2. Pathway of cyclooxygenase (COX) activity and the potential link to carcinogenesis.

[53]. It has also been shown that the intensity of immunoreactivity of COX-2 correlates with tumour grade [54].

Of particular interest, increased immunostaining for COX-2 has been observed in most HGPIN [55] and PIA lesions [52]. Recent data suggests that there is no difference in expression between normal, HGPIN and cancerous lesions, except in high grade cancers and PIA lesions, in which COX-2 expression was found to be significantly increased [52]. This data emanates from the use of validated immunohistochemical assays and quantitative RT-PCR. With regard to preclinical comparison, COX-2 expression has been found to be significantly increased in HGPIN lesions compared with normal prostate tissue in the TRansgenic Adenocarcinoma of the Mouse model of Prostate cancer (TRAMP). Animals in this model invariably develop HGPIN lesions followed by malignant changes, thus it may represent a suitable pre-clinical model to test chemopreventive strategies using COX-2 inhibitors [56]. In contrast to actual prostate tissue specimens, human prostate cancer cell lines, commonly used in mechanistic studies, such as PC3, LNCaP and DU145 do not express COX-2 [57].

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, sulindac and indomethacin, inhibit the activity of both COX-1 and COX-2; consequently, they can cause platelet dysfunction, gastrointestinal ulceration and renal damage. For this reason, selective COX-2 inhibitors, such as celecoxib and rofecoxib,

may be more attractive as potential chemopreventive agents, although their potential toxicities cannot be disregarded [58]. Epidemiological observations support the importance of NSAIDs in the chemoprevention of colon cancer [59]. However, epidemiological evidence for a protective effect of NSAIDs against prostate cancer is equivocal [60,61]. In vitro work using LNCaP and PC3 cell lines show that NSAIDs such as sulindac derivatives and etodolac decrease cell growth and induce apoptosis [62,63]. In contrast, apoptosis was not demonstrated in normal prostatic epithelial (PrEC) and stromal (PrSC) cell lines [62,63]. In vivo studies have shown that selective COX-2 inhibitors can induce apoptosis in PC3 cells grown in nude mice [64]. However, it is important to recognise that some of the effects of NSAIDs and selective COX-2 inhibitors are independent of their inhibition of COX-2 activity. Indeed, derivatives of celecoxib which lack COX-2 inhibitory activity can induce apoptosis in PC3 cells [65]. Multiple events independent of COX-2 include the induction of apoptosis, linked to the dephosphorylation of Akt and MAP kinase Erk2 [66]. In randomised trials, celecoxib has been shown to reduce the incidence of colonic and duodenal polyps in patients with familial adenomatous polyposis [67,68]. The clinical role of selective COX-2 inhibitors in the potential reversal or delay of progression of the pre-malignant lesion, HGPIN, is not yet known.

COX-2-derived metabolites may also have neoplastic promoting effects that are independent of ROS generation. COX-2 overexpression that may contribute to the malignant phenotype include decreased E-cadherin expression with consequent loss of cell-to-cell adhesion, matrix-metalloproteinase overexpression with an associated increase in invasiveness, and modulated production of angiogenic factors by cancer cells [69,70]. A correlation has been found between hypoxia-induced COX-2 expression and upregulation of vascular endothelial growth factor (VEGF) in PC3 and LNCaP cells [71]. Moreover, the COX-2-dependent effect of VEGF has been found to be inhibited by treatment with a specific COX-2 inhibitor, and this inhibitory effect was reversed by PGE₂ treatment [72]. Since VEGF plays an important role in angiogenesis, its upregulation by COX-2 expression and inhibition suggests an important role for COX-2 in angiogenesis.

6.2. Lipo-oxygenase enzymes

Lipo-oxygenases (LOX) are lipid peroxidising enzymes. In mammalian cells, the three major families are 5-, 12- and 15-LOX's [73]. The metabolites of arachidonic acid generated as a result of LOX catalysis, have been linked to prostate cancer development and progression [74]. In particular, it has been shown that inhibition of 5-LOX triggers rapid and massive apoptosis in PC3

and LNCaP cells [75]. This effect was blocked by the treatment of these cells with the anti-oxidant, *N*-acetyl-L-cysteine, but not by androgens, a powerful survival factor in prostate cancer cells.

15-LOX has two major forms, 15-LOX-1 and 15-LOX-2. These isozymes have different tissue distributions: the former are found in reticulocytes, macrophages and eosinophils; the latter are found in prostate, lung and skin [76,77]. The metabolites of these two enzymes have opposing effects, 15-LOX-1 is found to have increased expression in prostate cancer compared with normal tissue, while 15-LOX-2 is found to have increased expression in normal and benign prostate tissue and decreased levels in prostate cancer and HGPIN lesions, as determined by immunohistochemistry [78]. Reduced expression of 15-LOX-2 was also found to correlate with grade of prostate cancer [79]. 15-LOX-1 is capable of generating lipid peroxidation products e.g. the mechanism whereby it functions in reticulocytes. Thus, a shift in expression from predominantly 15-LOX-2 to 15-LOX-1 may serve as a biomarker for prostate cancer development and may represent a target for future chemoprevention strategies.

7. The role of androgens

Androgens play a key role in the development and progression of most prostate cancers [28,80]. Prostate cancer rarely develops in men castrated before puberty [81]. Indeed, it has been suggested that the increased risk of prostate cancer development and mortality among African Americans compared with Caucasian Americans may, in part, be related to elevated chronic androgen exposure in the former group [82].

There is increasing evidence to support the hypothesis that the role of androgens in prostate carcinogenesis is, at least in part, due to oxidative stress. The ROS induced by androgens may directly or indirectly result from their influence on mitochondria [83]. Experimental work carried out on the androgen-sensitive human prostate cancer cells, LNCaP, found that stimulation with physiological levels of androgens resulted in increased levels of ROS [15]. This hypothesis has been further supported by a failure to observe increases in ROS with androgens in the presence of the competitive androgen receptor antagonist, flutamide [16]. As one would expect, androgen stimulation of the cell lines, DU145 and PC3, which do not express functional androgen receptors, does not appear to influence the levels of ROS.

Increased COX-2 activity may lead to an increase in ROS [84]. Immunohistochemical studies, Western and Northern blots in human foetal ejaculatory ducts [85], and the distal vas of adult rats [86], have suggested that COX-2 expression is androgen-regulated. However,

COX-2 was not induced in LNCaP and PC3 prostate cancer cell lines following treatment with androgens [87]. Although definite conclusions cannot yet be stated, COX-2 expression may be influenced by androgens.

Although the first results of the Prostate Cancer Prevention Trial (PCPT) are now available, the potential role of anti-androgen therapy in the chemoprevention of prostate cancer is still not clearly defined. The PCPT was a large randomised, placebo-controlled trial of androgen manipulation in men at risk of developing prostate cancer. The trial recruited 18882 men, aged 55 years and above with a normal digital rectal examination (DRE) of the prostate and a serum PSA < 3.0 ng/ml. Men in the treatment group received the 5- α reductase inhibitor, finasteride (5.0 mg daily) and participants in the control arm of the trial received a placebo. All men were screened annually by serum PSA level and DRE. The primary endpoints to be studied were survival and the histological diagnosis of cancer in sextant biopsies of the prostate gland. The trial was terminated earlier than scheduled due to a 25% reduction in the prevalence of prostate cancer in the treatment group [88]. However, the trial showed a marginal, but significant increase in high-grade tumours in the finasteride group. This trial highlights the need to develop biomarkers and better strategies for assessing the potential benefit or detriment of the intervention being studied in any given trial.

8. Dietary anti-oxidants

Epidemiological, experimental and clinical studies implicate the potential benefits of a number of dietary anti-oxidants in the chemoprevention of prostate cancer [89]. Of these, vitamin E, selenium, lycopene and isoflavones found in soy beans have anti-oxidant properties, and vitamin E and lycopene are free-radical scavengers in the extracellular environment. Moreover, long-term administration of vitamin E decreases serum androgen concentration, which may contribute to its mode of action in the prevention of prostate cancer [90]. Vitamin E has also been shown to mediate a G1/S-phase arrest as a consequence of the decreasing expression of cell cycle regulatory proteins, such as cyclin D1, D3 and E, cyclin-dependent kinases -2 and -4, and upregulation of p27 [91]. Although the biochemical functions of selenium remain largely unknown, the anti-oxidant enzyme glutathione peroxidase is selenium-dependent [92]. Recently, it has been shown that selenomethionine can activate p53 by a redox mechanism that is independent of DNA damage, thus leading to the augmentation of DNA repair [93]. As well as having anti-oxidant actions, genistein (the principal isoflavonoid component of soy) inhibits tyrosine kinases and increases cell adhesion

[94]. Genistein also induces glutathione peroxidase in LNCaP and PC3 cells [95].

9. ROS and chemoprevention for prostate cancer

There is a growing body of evidence suggesting a role for oxidative stress in the pathogenesis of early prostate cancer. The long latency period in the development of prostate cancer provides a large 'window of opportunity' for intervention during this process, ideally suited to dietary manipulation or chemoprevention using diet-derived agents [41]. Biomarkers for assessing the efficacy of agents, such as oxidative DNA adducts to study the pharmacodynamic effects of vitamin E, may be of value in reducing the time-frames required for chemoprevention trials and for providing measurable indicators of potential benefit [41].

An example of one such trial is that of tomato sauce supplements prior to radical prostatectomies (see below). Such trials will be of increasing value in the design of large-scale studies, such as the ongoing Selenium and Vitamin E Cancer Prevention Trial (SELECT). This double-blind, randomised, placebo-controlled trial is aiming to recruit 32400 men, into four study arms (daily selenium + placebo, vitamin E + placebo, selenium + vitamin E, or placebo + placebo). The trial is expected to take 12 years to complete. The principal endpoint measured will be the incidence of prostate cancer by routine clinical care [96].

Although, the PCPT and SELECT trials have clearly defined endpoints, such as prostate cancer incidence, the endpoints chosen as the primary goals require large samples sizes and long time-frames. There are a number of inherent problems encountered in designing trials over smaller time-frames. Intermediate endpoints must be used for such trials, such as changes in histological pre-malignant lesions, biochemical and molecular markers. Such intermediate markers need to have the following characteristics, detectable in small specimens (prostate biopsies), high sensitivity and specificity and low range of spontaneous change [97]. Unfortunately, no reliable biomarkers have so far been validated to the extent that they can be used in large-scale trials to complement or even replace definitive endpoints such as cancer incidence. For example, HGPIN lesions have been targeted using chemopreventive agents. However, the effect of sampling error has not been fully considered. If patients with such lesions are to be recruited to clinical trials then repeat biopsies should be obtained to reduce sampling bias to a statistically acceptable level [98]. Unfortunately, the natural history, and consequent sampling error associated with HGPIN lesions, make this endpoint marker more appropriate in trials lasting years rather than months.

10. Oxidative DNA adducts as potential markers of oxidative stress

Oxidative DNA adducts are potential biomarkers of oxidative stress [11]. Oxidative DNA adducts, such as 8-oxo-dG, are formed by the direct reaction of hydroxyl radicals with DNA. In contrast, lipid peroxidation initiated by less reactive free-radicals, generate genotoxic ROS, such as peroxy and alkoxy radicals. Oxidative DNA adducts arising from lipid peroxidation include base adducts formed with malondialdehyde (MDA). Site-specific mutagenesis studies suggest that mutation frequency caused by indirectly formed oxidative DNA adducts, such as the MDA-guanosine adduct, is similar to that of directly formed oxidative DNA adducts, such as 8-oxo-dG [99].

A recent phase II trial was performed in 32 men undergoing radical prostatectomies for prostate cancer, who received supplements of tomato sauce (containing high levels of the anti-oxidant, lycopene) for three weeks prior to surgery [100]. Pre- and post-treatment levels of 8-oxo-dG were determined in blood leucocytes and prostate tissues. Post-treatment levels of 8-oxo-dG were significantly reduced in both prostatic tissue and leucocytes. At the study endpoint, levels of 8-oxo-dG were higher in prostatic tissue than in blood leucocytes, there was a correlation between prostate and leucocyte oxidative DNA damage. 8-oxo-dG levels were measured in prostate biopsies (not cancer cells specifically), so it is unclear whether levels are likely to be representative of normal, HGPIN or cancer cells. The results of this small study suggested that it may be possible to use blood leucocytes as surrogate markers for oxidative DNA damage in prostate tissue to monitor the efficacy of dietary anti-oxidant intervention over a short time-frame. An important issue to consider in the measurement of 8-oxo-dG levels is the artifactual oxidation of DNA both during the harvesting and preparation of tissue samples for analysis, which may lead to falsely high levels of 8-oxo-dG [101]. For discussion of these issues and the implications for validation, the reader is referred to a recent review [102].

11. Conclusions

Oxidative stress and COX-2 activity appear to play a role in the development and progression of prostate cancer. Further work carried out on ROS and COX-2 activity, particularly in the putative precursors of prostate cancer, such as PIA and HGPIN, will add to our understanding of the pathogenesis of prostate cancer and may allow these indices to be used as biomarkers of the efficacy of intervention in the chemoprevention of prostate cancer. Since agents currently exist that interfere with these cellular processes, such work may accelerate the

potential for the clinical use of anti-oxidants and/or COX-2 inhibitors in men with pre-malignant lesions, providing exciting opportunities for chemoprevention trials.

Conflict of interest statement

None declared.

Acknowledgement

SKP, RAS, AJG and JKM are supported by the Prostate Research Campaign UK. RAS, WPS and AJG are supported by Medical Research Council (MRC) (UK), the US National Cancer Institute and the University Hospitals of Leicester. JKM is supported by Cancer Research UK.

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Chemoprevention of prostate cancer by diet-derived antioxidant agents and hormonal manipulation (Review)

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Received August 2, 2002; Accepted September 16, 2002

Abstract. Cancer of the prostate is the most commonly diagnosed solid malignancy and the second leading cause of cancer-related death in men living in developed countries. With an ageing population, the number of men living with early stages of prostate cancer is expected to increase. There is an increasing need to prevent the onset of cancer or delay the progression of carcinogenesis in this organ. Chemoprevention is the administration of pharmacological agents to prevent, delay or reverse carcinogenesis. An example is the reversal of high grade intraepithelial neoplasia by hormonal manipulation using anti-oestrogens in breast carcinogenesis or anti-androgens in prostate carcinogenesis. Epidemiological data showing ethnic and geographic variations in the incidence of, and mortality from, prostate cancer have suggested that the consumption of certain dietary factors, particularly anti-oxidants, may be protective. These factors include the vitamins D and E, soy, lycopene and selenium. The administration of 5- α reductase inhibitors to patients with benign prostatic hyperplasia may also constitute a potentially chemopreventive intervention. The efficacy of chemopreventive agents needs to be investigated in randomised, placebo-controlled trials in suitable cohorts of high-risk individuals. In parallel, reliable assays of potential biomarkers of the efficacy of intervention need to be developed and validated rigorously.

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1. Introduction

It has been estimated that prevention of only 20% of cancers in a developed country such as the USA, would result in a saving of over \$21 billion in direct costs alone each year (1). Currently in the developed world, cancers that are responsive to hormonal treatment account for almost half of all newly diagnosed female cancers and about a quarter of all newly diagnosed male cancers (2). Based on the large-scale success of hormonal manipulation using tamoxifen during late and early stages of breast carcinogenesis (see below), there is currently optimism for similar strategies in the chemoprevention of other hormone-related solid malignancies.

Cancer of the prostate is the most commonly diagnosed solid malignancy and the second leading cause of cancer-related death in men living in developed countries (3). Although the exact cause of prostate cancer remains unclear, there are factors that appear to be linked to its aetiology. The incidence of prostate cancer increases with age (4); as the subpopulation of older individuals increases, the incidence and prevalence of prostate cancer are expected to increase.

It has been recognised for almost half a century that familial aggregation, the occurrence of multiple cases of prostate cancer within a family, is thought to account for approximately 10% of all cases of prostate cancer. Genetic factors as a whole may account for as much as 42% of prostate cancer risk (5). To date, six genetic loci have been described that are relevant to hereditary prostate cancer (6).

The tremendous variation in the incidence of, and mortality from, prostate cancer across ethnic and geographic groups, coupled with changes in risk among migrants, have stimulated the search for environmental factors involved in prostate carcinogenesis (7). In particular, dietary micronutrients and antioxidants are under intense scrutiny. There is increasing evidence that diet may play a significant role in early prostate carcinogenesis (8). This evidence base provides the rationale for potential intervention strategies to prevent or delay the onset of prostate cancer. The chemoprevention of cancer is a relatively new concept, defined as the administration of pharmacological agents (drugs or diet-derived supplements) to prevent, delay or reverse carcinogenesis (9).

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Key words: biomarker, carcinogenesis, prevention, oxidation

2. Prostate carcinogenesis

The prostate is a tubuloalveolar gland that surrounds the urethra and encases the ejaculatory ducts. It is adjacent to the seminal vesicles. The gland consists of smooth muscle stroma and acini lined by a bilayered epithelium, composed of basal cells and mature columnar secretory cells. The secretory cells contribute many components to seminal plasma including prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA). Increases in gland weight after 30 years of age, which are nearly ubiquitous among western populations, are largely due to stromal and epithelial hyperplasia. Benign prostatic hyperplasia (BPH) is a nodular disease of heterogeneous composition, and may include smooth muscle hypertrophy. Local consequences include infarction, atrophy and inflammation. Although controversial, there is no consensus of opinion to suggest that BPH is a premalignant condition.

In contrast, high grade prostatic intraepithelial neoplasia (HGPIN) is a lesion that harbours genetic and cellular changes similar to those found in prostate adenocarcinoma, without invasion of the basement membrane. The evidence that HGPIN is a precursor of prostate cancer is extensive (10,11). HGPIN shares a number of similarities with prostate cancer in terms of spatial distribution and architectural characteristics (12). The prevalence of both HGPIN and prostate cancer increases with age (13). According to one survey, HGPIN is more frequently found in prostate glands containing cancer (83%) than in benign glands (43%) (14). Cytological characteristics are very similar to prostate cancer but with less variability in nuclear size, enlargement of nuclei and prominent nucleoli. Anatomically, HGPIN is frequently found in the peripheral zone of the prostate gland (15,16), the site at which 70% of prostatic carcinomas occur.

The vast majority of prostate cancers are adenocarcinomas. Historically, screening for prostate cancer relied on digital rectal examination (DRE), but over the past two decades, the detection of prostate cancer has been augmented by measurement of serum PSA level. Indeed, the availability of this biochemical assay may account for the significant increase in the incidence of prostate cancer that occurred in the USA from 1989 to 1992 (17). Approximately one third of men with PSA values above 4.0 ng/ml will have cancer (18). PSA levels may be transiently elevated after prostatitis, urethral manipulation, prostatic biopsy and possibly ejaculation (19). The half-life of PSA in the plasma is 2.2-3.2 days (20). Although PSA-detected cancers are usually clinically confined to the prostate, up to 40% of such cases may have evidence of extraprostatic cancer extension if the prostate is removed surgically and examined pathologically (21). In patients with BPH, serum PSA levels can be halved by treatment with the anti-androgen, finasteride (22). Although monitoring serum PSA is well established in the management of prostate cancer and is of some use in the early detection of cancer limited to the prostate gland (23), it lacks specificity and is likely to be of highly limited use as a surrogate biomarker of intervention during preneoplastic stages of carcinogenesis.

Molecular mechanisms responsible for the initiation and progression of prostate cancer have not been fully elucidated.

Strong evidence supports the theory that prostate carcinogenesis proceeds through a series of genetic alterations rather than a single genetic event. This multistep requirement appears to involve the activation of oncogenes, such as *c-myc*, *ERBB2*, and *BCL2*, and the inactivation of tumour suppressor genes, such as *TP53*, *PTEN*, *p16* and *p27* (24). The most common genetic alterations in prostate cancer cells are losses and gains in chromosomal arms, particularly losses in 8p, 10q, 13q and 16q and gains in 7p, 7q and 8q (reviewed in ref. 25).

Prostate cancer has long been recognized as an androgen dependent tumour. Androgens play a critical role in the development of prostate cancer by acting as mitogens (26). Androgen signalling is mediated through the androgen receptor (AR), a member of the nuclear receptors that are ligand dependent transcription factors. Mutations in the AR gene are not uncommon in metastatic prostate cancer (27), and may increase the sensitivity of cells to androgens (28). Manipulation by anti-androgen therapy plays an important role in the treatment of BPH and early prostate cancer.

3. Potential for chemoprevention

The first large-scale clinical trial to demonstrate success from a chemopreventive intervention was the Breast Cancer Prevention Trial study (29). This trial involved 13,388 women of 'relatively high risk' (i.e. with a substantially greater than 1.66% chance of developing breast cancer within 5 years), but free of detectable invasive breast cancer at study recruitment. The anti-oestrogen tamoxifen reduced relative risk for subsequent development of breast cancer in the treated group as a whole by 45%, and may also have prevented the progression of established preneoplastic lesions. The positive results of this detailed study, using a familiar and well described drug, allowed some degree of quantification of the likelihood of benefit in this group of subjects, in whom the balance between potential toxicity and potential benefit is critical in clinical decision making. The results of this trial also led the US Food and Drug Administration to approve the use of tamoxifen by women deemed at increased risk of developing this disease. Under these circumstances, accurate assessment of an individual's risk of developing cancer is also essential to the decision whether to treat with tamoxifen or not; research into this area of molecular epidemiology must be paramount, occurring in parallel to the advancement of potentially chemopreventive compounds. Based on preliminary findings (see below), it is conceivable that hormonal manipulation during prostate carcinogenesis may be the next success story for cancer chemoprevention.

Although the molecular events associated with early prostate carcinogenesis are not fully understood, it is widely accepted that prostate carcinogenesis is a multi-step process of long duration (see above). Although there is no consensus on exact estimates of doubling times for prostate cancers, most clinicians would predict doubling times of 2-6 years for most prostate adenocarcinomas (30). Carcinogenesis prior to this histological diagnosis is also likely to have taken many years, if not decades. It has been suggested that HGPIN may take 20-30 years to develop and that progression to clinically significant cancer may take between 3-15 years (31).

As discussed above, HGPIN is a reversible condition that represents an early stage of prostate carcinogenesis. It also represents a potential target and biomarker for chemopreventive intervention (Fig. 1). Reversibility of HGPIN has been shown by androgen deprivation in a case-control study using multi-agent hormonal manipulation prior to prostatectomy (32). A more recent case control study of the effect of complete androgen deprivation on localised prostate cancer for at least 3 months prior to prostatectomy has found that tumour volume may be reduced significantly in the treatment group compared to control men, suggesting greater resectability (33). The importance of considering, within the definition and cost effectiveness of cancer chemoprevention strategies, a delay in cancer onset as a valid endpoint in patients with intraepithelial neoplasia (IEN) has recently been argued by the IEN Task Force of the American Association for Cancer Research (34).

4. Antioxidation as a chemopreventive mechanism

There is strong evidence from pre-clinical models and human tissue implicating reactive oxygen species (ROS) as potential targets during prostate carcinogenesis. ROS, which may be generated endogenously or exogenously, are associated with carcinogenesis and cancer progression in many tissues (35). ROS are capable of causing oxidative damage to important cellular molecules, such as lipids, proteins and both nuclear and mitochondrial DNA. Like the incidence of prostate cancer (3), increased production of ROS and decreased levels of antioxidants and antioxidant-generating enzymes appear to be age-dependent (36).

At least 75% of cases of metastatic prostate cancer are androgen-dependent at initial diagnosis (37); physiological and supraphysiological levels of androgens alter the pro-oxidant/antioxidant balance of prostate cancer cells towards the pro-oxidant state, as shown *in vitro* by exposure of human prostate cancer cells to increasing concentrations of androgens (38). An elevation of mitochondrial activity was suggested by greater oxygen consumption and increases in lipid peroxidation were also measured. This study supports the concept that androgens increase oxidative stress. Androgens also alter intracellular levels of glutathione, a scavenger of ROS, and certain detoxification enzymes important in protecting the cell from the protein and DNA damaging effects of ROS (38).

Inflammatory cytokines upregulated in the early stages of prostatic carcinogenesis can generate ROS *per se* (39,40) or lead to upregulation of enzymes such as cyclooxygenase-2 (COX-2), also recently linked to prostate cancer (41). COX-2 catalyses the production of prostanoid hydroperoxides that may in turn act as a source of ROS (42). Glutathione S-transferase (GST) P1 is a detoxification enzyme that catalyses the conjugation of potentially damaging radical species to glutathione. Inactivation of the *GSTP1* gene has been found in nearly all human prostate cancers analysed (reviewed in ref. 43), as also appears to be the case for HGPIN, thus rendering prostate cells vulnerable to the damaging effects of ROS. It is therefore conceivable that ROS play a part in the development of prostate cancer from its earliest stages and that intervention with antioxidants may combat this disease.

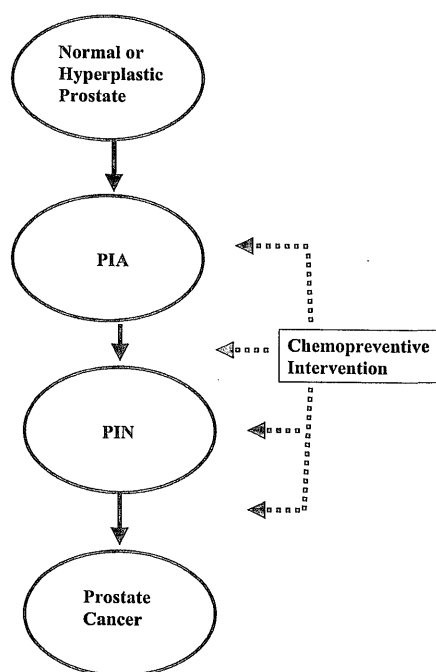


Figure 1. Simplified pathway of prostatic carcinogenesis. Normal prostatic epithelial cells may take up to 30 years to develop proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN), which may then take between 3-15 years to develop clinically significant prostate cancer (see text). The potentially reversible stages PIA and PIN may represent a continuum (94), although it has been proposed that carcinoma can develop directly from PIA (95).

5. Chemopreventive agents in development

A number of agents have been considered as potential diet-derived or pharmacological agents for prostate cancer chemoprevention. They include the vitamins, A, C, D and E, soy constituents, lycopene, selenium and 5- α reductase inhibitors. With regard to dietary intervention, fat intake may also be of relevance. It should be noted that many of the agents described in more detail below exert their effects as anti-oxidants. Therapeutic intervention with synthetic drugs, in particular 5- α reductase inhibitors and tyrosine kinase inhibitors, may complement dietary supplementation strategies. Current large-scale clinical trials of prostate cancer chemoprevention ongoing in the USA are listed in Table I.

Vitamin E. Vitamin E is the general term for all tocopherols and tocotrienols, of which α -Tocopherol is the most biologically active form. Although γ -tocopherol makes a significant contribution to the vitamin E content of foods, α -tocopherol is the most effective chain-breaking and lipid-soluble antioxidant. α -Tocopherol is found in vegetable oils,

Table I. Prostate cancer chemoprevention trials currently sponsored by the US National Cancer Institute.^a

Phase	Agent(s)/drug(s) under investigation	Principal
I	Celecoxib	M. Carducci (MD, USA)
I	Lycopene	D. Gustin (IL, USA)
II	Soy	A. Venook (CA, USA) I. Berkowitz (DW, USA) G. Fleming (IL, USA) D. Hurd (NC, USA)
II	Doxercalciferol	M. Cohen (IW, USA) W. See (WI, USA) G. Wilding (WI, USA)
II	Flutamide/Toremifene	J. Nelson (PA, USA)
IIIB	Difluoromethylornithine	T. Ahlering (CA, USA)
III	Selenium	D. Jarrard (ECOG, USA) J. Marshal (SWOG, USA)
III	Selenium/Vitamin E	D. Karp (ECOG, USA) E. Klein (SWOG, USA)
III	Finasteride	C. Coltman (SWOG, USA)

^aApart from the clinical trial of finasteride, all the clinical trials shown were recruiting patients at the time of publication of this article. ECOG, Eastern Cooperative Oncology Group. SWOG, Southwest Oncology Group. Information obtained from the website of the US NCI (<http://www.nci.nih.gov/>).

seeds and nuts. It is now widely accepted that vitamin E is a major anti-oxidant in cells, capable of inhibiting lipid peroxidation, with the potential for a wide range of anti-cancer properties (44,45).

Experimental evidence has suggested that vitamin E may play a role in reducing the risk of developing a range of cancers including prostate cancer (46). Observational studies have shown the association of vitamin E and prostate cancer risk. A study of 2,974 men with a 17-year follow up indicated that low serum vitamin E levels are associated with a higher risk of prostate cancer (47). *In vitro* work carried out on human prostate cancer cell lines, have shown that vitamin E inhibits the growth of these cells (48). Indeed, a recent study showed that supplements of vitamin E can inhibit high-fat promoted tumour progression in nude mice bearing LNCaP xenografts; this effect may relate to the anti-oxidant properties of vitamin E (49). LNCaP cells were originally established from androgen sensitive human prostate cancer cells derived from a lymph node metastasis (50).

Preclinical data suggesting chemopreventive potential have resulted in vitamin E being tested in a large-scale clinical trial in individuals at increased risk of developing lung cancer. The α -tocopherol and β -carotene (ATBC) study was a double-blind randomised, controlled trial of 29,133 male smokers. Lung cancer incidence was the principal endpoint measured. Paradoxically, the trial showed an increase in lung cancer risk among a subset of men given β -carotene supplements who continued to smoke at least twenty cigarettes per day and who also had a high intake of alcohol recorded. However, secondary analysis of 6-year follow-up data demonstrated a 32% reduction in the incidence of prostate cancer and a 42% reduction in mortality from this disease among men who received supplementary vitamin E (51). Based on these findings, the role of vitamin E in prostate cancer prevention appears promising and is now being investigated further in the Selenium and Vitamin E Cancer Prevention Trial (SELECT) (see below).

Selenium. Selenium is an essential trace element in the diet. Although the mechanism of action is not fully understood, selenium can act as an anti-oxidant (52). Glutathione peroxidase, an enzyme that protects the cell from oxidative damage is known to be selenium-dependent (53). *In vitro* studies have shown that selenium has inhibitory effects on DU 145 cells (54). DU 145 cells represent an androgen insensitive human prostate cell line established from cancer cells taken from a bone metastasis (55).

Similar to the secondary analysis of the ATBC trial data, the effect of selenium in reducing the risk of prostate cancer has been discovered by secondary analysis of data from a trial whose primary endpoint focussed on another solid malignancy. In a randomised, placebo-controlled study of 1312 patients with non-melanoma skin cancer, selenium was found to significantly decrease prostate cancer incidence (56). Indeed, the reduction in the incidence of prostate cancer in the group receiving supplements of selenium was as high as 3-fold. Selenium and vitamin E's potential beneficial effects with regard to prostate cancer incidence have been secondary findings from the two clinical studies mentioned and are therefore open to criticism. One subsequent study has addressed the relationship between selenium acquired in the diet and the risk of prostate cancer: A large nested case control study (within the context of the Health Professionals Follow-Up study) has shown that increased selenium levels measured in toe-nail chippings was associated with a decreased risk of prostate cancer, after controlling for family history of prostate cancer, calcium and lycopene intake (57). Toenail selenium levels reflect selenium intake integrated over many months and therefore act as a useful indicator of chronic selenium intake.

The magnitude of the changes in incidence seen in the trials described above have been deemed promising enough to justify an extremely large prospective study of prostate cancer chemoprevention. SELECT has commenced under the co-ordination of the South West Oncology Group (SWOG), San Antonio Cancer Institute, Texas. This double-blind randomised, placebo-controlled trial aims to recruit 32,400 men, who will receive daily supplements of a combination (as shown in Fig. 2) of two of the following: Selenium (200 μ g),

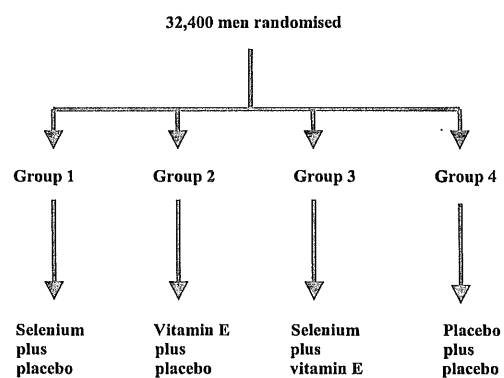


Figure 2. Design of the SELECT clinical trial. For supporting refs., see text.

vitamin E (400 mg) or placebo. The study is expected to take 12 years to yield meaningful data regarding the significance of vitamin E, selenium and the combination of the two in preventing prostate cancer. The principal endpoint of the trial is the incidence of prostate cancer, assessed by routine clinical care including annual DRE and serum PSA measurement. In addition, quality of life will be assessed, and blood and prostate tissue samples stored for analysis of micronutrient levels and certain biological and genetic biomarkers of prostate cancer risk. The incidence of lung and colorectal cancers will also be studied.

The long timeframe projected for the SELECT demonstrates an important issue in the advancement of potential chemopreventive agents from preclinical data to large-scale clinical trials. In light of the long natural history of prostate carcinogenesis (see above), if the incidence of cancer acts as the only endpoint measured in chemoprevention trials, prolonged time periods will be necessary to obtain results. More importantly, during this prolonged period of time, no indication will be available on the potential efficacy of the intervention, nor regarding harm or benefit caused to participants. Biomarkers of carcinogenesis, suitable to the agent under investigation, are therefore essential in the clinical development of chemopreventive agents. An example of such a biomarker is measurement of oxidative DNA adduct levels in prostate tissue or in white blood cells as a 'surrogate' for the prostate (58), to look for antioxidant changes elicited by dietary supplementation with selenium. Criteria that should be used in the method of selection of such biomarkers have been reviewed in the chemoprevention of colon cancer (59), and the same principles are applicable to the chemoprevention of prostate cancer.

Lycopene. Lycopene is the predominant carotenoid present in tomatoes. It is also the predominant carotenoid found in certain fruits, such as papayas, grapefruit and the edible fruit from the Autumn olive tree. In most communities, tomatoes constitute the major dietary source of lycopene intake. Lycopene's anti-oxidant properties are characterised by its

capacity for scavenging free radicals (60). As discussed above, this mechanism may reduce the risk of cancer by protecting DNA and membrane lipids from oxidation by free radicals.

A number of epidemiological studies have suggested that lycopene may be beneficial in reducing the risk of developing prostate cancer. The initial study of this type reported a 50% lower risk for the highest versus the lowest quartile of serum lycopene. However, this study incorporated only 103 case-control patients and the results were not found to be statistically significant (47). A subsequent cohort study identified that increased tomato consumption was associated with a statistically significant reduced risk of prostate cancer in Adventist men (61). More recently, a large prospective cohort study has found that consumption of lycopene is inversely related to the risk of prostate cancer, especially for aggressive disease (62).

Based on the above findings, a randomised, clinical trial was conducted with lycopene supplements twice daily in 26 men with early prostate cancer for a period of three weeks prior to radical prostatectomy. The design of the study could be described as Phase IIa; the treatment group contained 15 patients and there were 11 patients in the control group. Statistical analysis has suggested a decrease in serum PSA and a decrease in diffuse histological involvement of the prostate by HGPIN in the treatment arm of the trial (63), and larger Phase II studies are planned. In a similar study published around the same time, the effect of a tomato sauce-based entrée was investigated as a dietary intervention in 32 men with localised prostate adenocarcinoma for 3 weeks (58). In addition to serum and prostate lycopene concentrations, levels of the oxidative DNA adduct, 8-hydroxy-deoxyguanosine (8-OH-dG), were measured in blood leukocytes and prostate tissue before and after the intervention. Significant decreases in 8-OH-dG adduct levels were observed in blood leukocytes and prostate tissue as a result of the intervention, of the order of 20% in the blood cells. A particularly interesting result was a correlation observed between adduct levels in blood leukocytes and prostate tissue, which offers some potential in the development of this biomarker as a surrogate measure of oxidative DNA damage in the target tissue.

Vitamin D. In humans, the physiologically active form of vitamin D is obtained principally in two ways. Firstly, the inactive form obtained from the diet becomes 'activated' following hydroxylation in the liver and kidney. Secondly, the inactive form can also undergo initial activation when the skin is exposed to ultraviolet range sunlight.

Although vitamin D is primarily involved in bone and calcium metabolism, an initial association of vitamin D with prostate cancer was suggested by an epidemiological study published in 1990. In this study, the mortality rates from prostate cancer increased as exposure to sunlight decreased (64). As it is known that sunlight is essential for the synthesis of the active form of vitamin D, the role of this in preventing prostate cancer was proposed. Following this study, *in vitro* experiments examining the effect of vitamin D on human prostate cancer cell lines have shown inhibition of growth (65,66).

The first clinical trial using vitamin D in patients with androgen-insensitive prostate cancer failed to show any significant response (67). In fact, hypercalcaemia was noted to be a major adverse effect. A more recent clinical trial has been carried out, using vitamin D supplements in 7 patients in whom early recurrence of prostate cancer following definitive treatment had occurred (68). Although the rate of rise of serum PSA decreased significantly, some of these patients developed hypercalcaemia. Analogues of vitamin D have been developed which appear to have potent anti-proliferative effects but less adverse effects in terms of hypercalcaemia (69). These analogues may play a role in future clinical trials of prostate cancer chemoprevention.

Dietary fat intake. A high level of dietary fat consumption is a characteristic of Western populations. The link between high dietary fat intake and increased mortality rates for prostate cancer has been observed since the early 1990s (70). Japan, for example, has a low dietary fat intake and one of the lowest incidences of prostate cancer in the world.

Essential fatty acids are unsaturated and can be categorised into two groups: n-6 fatty acids, found in vegetable oils, and n-3 fatty acids, found in fish oils. Experimental data suggest that n-3 fatty acids inhibit the growth of human prostate cancer cell lines, whereas n-6 fatty acids stimulate proliferation (71). One population-based case-control study in Auckland, New Zealand during 1996-1997, involving 317 prostate cancer cases and 480 age-matched community controls, found that erythrocyte levels of long-chain n-3 polyunsaturated fatty acids (derived from marine foods) were associated with a reduced risk of developing prostate cancer (72).

The mechanism of action of high dietary fat intake's potential association with prostate cancer is not clear. Fatty acids are known to be precursors in the biosynthesis of prostaglandins and androgens, both implicated in prostate carcinogenesis. Prostaglandin E₂, the principal product of cyclooxygenase-2 catalysis, has been shown to promote the growth of prostate cancer cells in tissue culture (73). Testosterone is converted to 5-dihydroxytestosterone by 5- α reductase. A study carried out in human prostate cancer cells grown in culture showed that this enzyme can be inhibited by n-3 fatty acids (74).

The reduction of animal fat intake is encouraged by most Western governments, as this may also be linked to the main causes of death in these countries, particularly cardiovascular disease. A reduction in dietary fat intake may be of benefit in reducing the risk of developing prostate cancer, but further clinical data are necessary to clarify this potential association. Appropriate trials may consist of dietary intervention, similar to studies performed recently in patients at risk of developing colorectal cancer (75). Such approaches may be used in combination with chemoprevention using pure diet-derived supplements, if certain fatty acids are found to confer protection within the context of a certain dietary matrix.

Soy products. Soy and soy products form a significant part of the diet in South-East Asia; countries which have a low incidence of clinical prostate cancer when compared with those in which Western diets are consumed (76). The principal isoflavonoid components of soy are genistein and daidzein.

Epidemiological studies have prompted experimental research to be carried out using these components. Genistein has been shown to induce apoptosis in and to inhibit the growth of prostate cancer cell lines (77). In animal models of prostate carcinogenesis, genistein has shown growth inhibitory effects (78). Genistein is known to exert its effects by a number of different mechanisms, including inhibition of tyrosine kinases, increased cell adhesion and antioxidation (79). Since genistein is a diet-derived product, it is anticipated that it should not demonstrate the adverse effects of synthetic drugs, at least at doses equivalent to those used in the diet. No chemoprevention trials of pure genistein have thus far been performed in humans.

5- α reductase inhibitors. Androgens play a key role in the development of the majority of prostate cancers. Epidemiological evidence in support of this contention is the observation that prostate cancer rarely develops in men castrated before puberty (80). It has also been suggested that the increased prostate cancer risk and mortality in African Americans compared to white Americans may in part be related to the elevated chronic androgen exposure in the former, although the exact nature of this exposure in terms of serum testosterone concentrations and endogenous androgen metabolism has not been fully elucidated (81).

Finasteride, a 5- α reductase inhibitor, may be an agent of potential use in the chemoprevention of prostate cancer. Its mechanism of action is the inhibition of the conversion of testosterone to dihydrotestosterone, an androgen ten times more potent in promoting normal and hyperplastic growth of prostate tissue (82). Experimental evidence has shown that growth of prostate cancer cell lines can be inhibited by 5- α reductase inhibitors such as finasteride (83,84). This effect has also been observed in animal models (85).

Based on preclinical data, a randomised, placebo-controlled trial, which commenced recruitment in 1993 and rapidly enrolled 18,882 men (aged 55 years and above with a normal DRE and a PSA <3.0 ng/ml) is ongoing (86). Patients in one arm of the trial are receiving finasteride (5 mg/day) and those in the other arm are receiving a placebo. All men are being screened annually with DRE and measurement of serum PSA. The primary endpoints are survival and histological diagnosis of adenocarcinoma in sextant biopsies of the prostate gland. The results of this trial, the Prostate Cancer Prevention Trial (PCPT), are expected in 2003. The outcome of this major study will provide crucial insights into the harm:benefit ratio of hormonal manipulation in healthy men at high risk of developing prostate cancer.

Other diet-derived agents. Epidemiological and experimental studies examining the role of other dietary nutrients, such as vitamin A and C, and other trace elements, particularly zinc and cadmium, have thus far been equivocal (87-89). There are currently no large-scale clinical chemoprevention trials of these agents planned.

6. Role of chemoprevention of prostate cancer

The particularly long latency period of prostate carcinogenesis provides a window of opportunity for intervention during this

process, ideally suited to chemoprevention using diet-derived agents or drugs that effect hormonal manipulation. No chemopreventive agent can be considered of proven value unless it has been tested in randomised placebo-controlled trials. At present, these trials are large, of long duration and extremely expensive. One of the reasons is that the principal endpoint used in Phase III trials is cancer incidence, as discussed above and illustrated in Table II. The development of biomarkers of the efficacy of intervention as well as of prostate carcinogenesis *per se* should encourage the advancement of agents at the Phase I-II clinical trial level. Assays for such biomarkers need to be of high specificity, sensitivity, reproducibility and practical for the analysis of large numbers of clinical samples. Currently, HGPIN is under intense genetic and molecular scrutiny as a histological stage that provides an opportunity for validating biomarkers of prostate carcinogenesis (90). The study of the role of oxidative species in prostate cancer may accelerate over the next decade (91) with the development of assays of the consequences of such species on cellular DNA and proteins (92).

Although results from some of the chemopreventive agents thus far tested are encouraging, present evidence for their widespread use is insufficient to make definite recommendations. The results of the two large-scale chemoprevention trials for prostate cancer are eagerly awaited. The first results of the PCPT trial are expected in 2003, and results from the SELECT trial about a decade later. These results may offer greater insight into prostate chemoprevention, and if the results are favourable, they will undoubtedly add further impetus to research into chemoprevention of prostate cancer.

On the other hand, if the results of these large-scale trials are negative, then questions will be asked about the choice of agents and the study design. Ultimately, prostate cancer chemopreventive agents may be administered to healthy men over a long period of time, and the safety of such agents should be established rigorously in Phase I-II trials. It is increasingly unacceptable to perform Phase III trials in healthy or 'at-risk' individuals without Phase I-II data regarding the potential toxicity of the particular formulation of the agent being tested. It is also being recognised widely that the clinical advancement of promising agents, such as vitamins D and E, soy, selenium and 5- α reductase inhibitors, must incorporate the measurement of biomarkers of efficacy in pilot clinical trials, exemplified by the recent advancement of the agent lycopene. Without such biomarkers, it will take a decade or longer for each trial to tell us whether we are moving forwards or backwards in preventing prostate cancer.

Acknowledgements

Our sincere thanks to Professor Andy Gescher for his detailed review of the manuscript and to Sue Spriggs for checking the accuracy of the references. SKP and RAS are supported by the UK Medical Research Council and the Prostate Research Campaign UK. J.K.M. is supported by Cancer Research UK.

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